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EFFECTS OF FOLLICLE-STIMULATING AND LUTEINIZING PITUITARY EXTRACTS ON THE OVARIES OF THE INFANTILE AND JUVENILE RABBIT[1]

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From the Zoological Laboratories, University of Wisconsin

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There have been presented recently practical methods for the substantial separation of the follicle-stimulating and luteinizing factors to be found in the mammalian anterior hypophysis. The present work is concerned with the action of these two factors upon the ovary of the sexually immature rabbit when administered in varying sequence and for varying periods of time. The luteinizing and follicle-stimulating preparations here employed are the same as those which were used in the work previously reported (Fevold et al., 1933).

A comparison of the response of juvenile and infantile rabbits. The young rabbit has been used comparatively little in the study of the gonadotropic properties of pituitary extracts. Siegmund (1930), Zondek (1931) and Clauberg (1932) have briefly described the effects of urinary preparations upon the young rabbit's ovary and have pointed out the essential similarity of the picture obtained to that observed in the rat and mouse. In addition, Clauberg has shown that rabbits weighing between 600 and 800 grams give little ovarian response to even excessive dosages of prolan, whereas rabbits between 1200 and 1400 grams in body weight readily respond.

Similarly, we have found that the female chinchilla rabbit, four weeks of age and between 500 and 700 grams in weight shows no ovarian response to a dosage of pituitary extract which is ample to elicit a maximal response in a rabbit of the same breed which is between twelve and thirteen weeks of age and weighs about 1300 grams. Moreover, a rabbit about ten weeks of age will give only a meagre response to a similar dosage. In agreement with Clauberg, we have chosen to speak of the younger non-responsive

[1] Aided by a grant from the National Research Council, Committee for Research in Problems of Sex.

rabbits as "infantile," and of the older rabbits as "juvenile" (tables 1 and 2).

A careful histological comparison of the "infantile" and "juvenile" type of ovary has brought to light a probable anatomical basis for the difference in their reactions to pituitary stimulation. The infantile ovary (fig. 1) is made up almost entirely of numerous primordial follicles separated from each other by very thin layers of connective tissue stroma. These primordial follicles are distributed uniformly about the comparatively small medullary portion of the ovarian stroma. It should be emphasized that at this stage no follicular antra have as yet been formed.

TABLE 1

*Infantile rabbits given follicular-stimulating and unfractioned pituitary extracts**

RABBIT NUMBER	BODY WEIGHT	AGE	TYPE OF EXTRACT
	grams	*days*	
33	700	56	UNF.
34	700	56	UNF.
35	700	50	UNF.
53	460	36	UNF.
59	470	37	F.S.
60	478	37	F.S.
69	490	44	F.S.
73	630	58	F.S.
116	500	42	F.S.
237	195	28	F.S.
238	254	28	F.S.
475	500	39	F.S.

* All animals given equivalent of 0.25 gram twice daily for 5 days except no. 475 which received twice this amount; observations made 24 hours after last injection.

UNF.—unfractionated extract.

F.S.—follicle-stimulating extract.

The ovaries of these animals remained unmodified (fig. 1).

The "juvenile" ovary, however, shows a much more highly advanced histological condition (fig. 2). The primordial follicles have been substantially reduced in number and are to be found only about the periphery of the gonad. Both the medullary and interstitial stroma are very much increased. A highly significant change is the development of numerous follicular antra.

It thus becomes apparent that under the conditions of the present experiments, the known gonadotropic pituitary hormones are able to affect only an antrum-containing follicle and are unable to stimulate a primordial follicle to development. The physiological factors responsible for conditioning a Graafian follicle so that it will respond to pituitary stimulation are unknown.

TABLE 2

Ovarian response of juvenile rabbits to purified follicle-stimulating and unfractionated pituitary extract

RABBIT NUMBER*	TYPE OF EXTRACT	OBSERVATIONS ON 5TH DAY OF TREATMENT	OBSERVATIONS ON 12TH DAY	OBSERVATIONS ON 18TH DAY	OBSERVATIONS ON 20TH DAY
284	F.S.	Clear cystic follicles; rt. ov. 2.3 × 0.7 × 0.7 cm.		Rt. ov. 1.6 × 0.3 × 4 cm. Ovary involuted	
262	F.S.	Clear cystic follicles; rt. ov. 1.6 × 0.5 × 0.8 cm.	Rt. ov. 1.35 × 0.4 × 0.5 cm. Follicles regressing		Rt. ov. 1 × 0.35 × 0.3 cm. Ovary involuted
261	F.S.	Clear cystic follicles; rt. ov. 1.7 × 0.7 × 0.8 cm.	Rt. ov. 1.7 × 0.4 0.4 × 0.4 cm. Ovary involuting		
264	F.S.	Clear cystic follicles; lt. ov. 1.5 × 0.5 × 0.6 cm.	Lt. ov. 1.2 × 0.35 × 0.35 cm.		Lt. ov. 0.9 × 0.3 × 0.3 cm. Ovary completely involuted
251	UNF.	Numerous c.l. and large foll.; rt. ov. 1.1 × 0.5 × 0.7 cm.	Rt. ov. 1.1 × 0.45 × 0.3 cm.; corpora are degenerated		
253	UNF.	Numerous c.l. and large foll.; rt. ov. 1.35 × 0.55 × 0.4 cm.; lt. ov. 1.6 × 0.6 × 0.6 cm.	Rt. ov. 1.3 × 0.25 × 0.5 cm. Lt. ov. 1.1 × 0.3 × 0.45 cm. Corpora are degenerated		
272	UNF.	Numerous c.l. and large foll.; lt. ov. 2 × 0.7 × 0.7 cm.		Lt. ov. 1.15 × 0.4 × 0.4 cm. Ovary involuted	

* All rabbits used weighed between 1200 and 1500 grams and were between 12 and 14 weeks old. The daily dose in all cases was the equivalent of 0.5 gram original dried tissue given in two injections. Observations made 24 hours after last injection.

F.S.—follicle-stimulating extract.

UNF.—unfractionated extract.

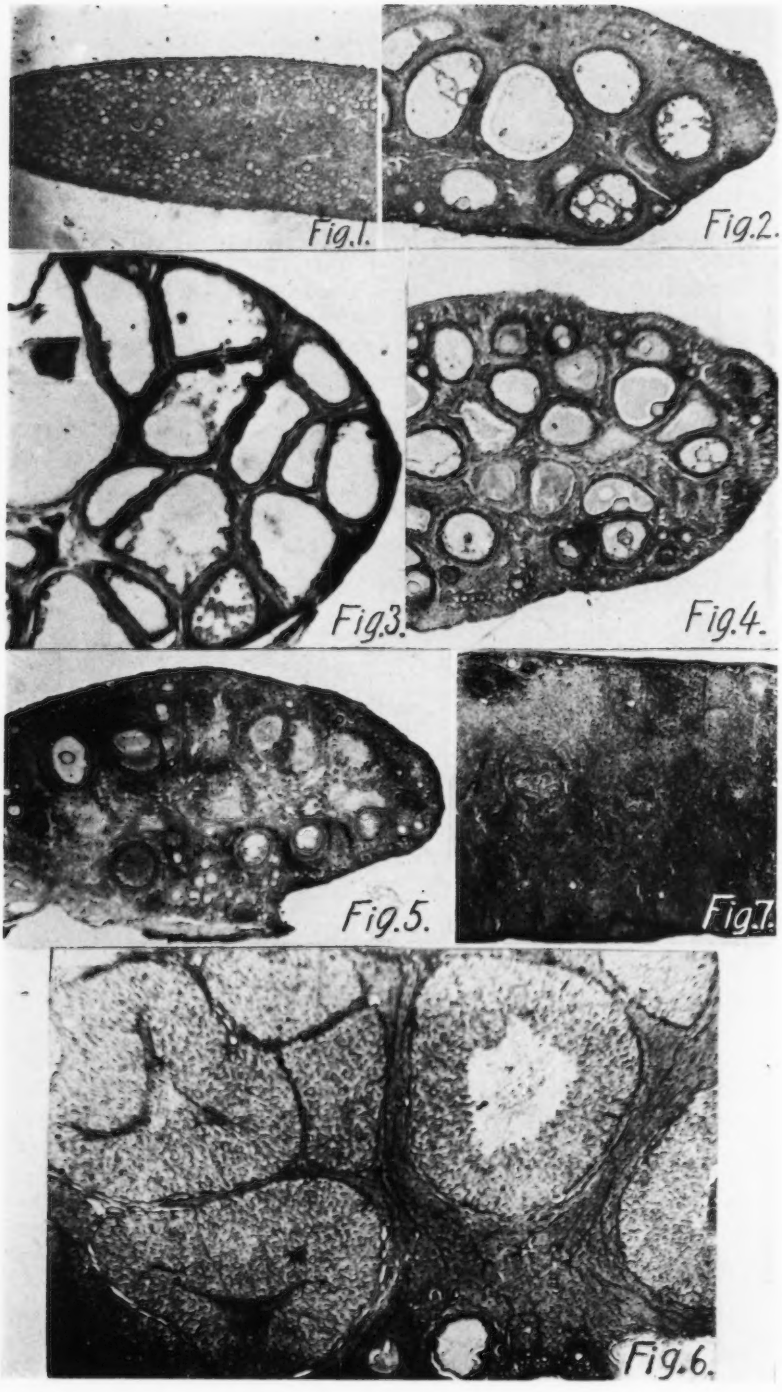
Fig.1.
Fig.2.
Fig.3.
Fig.4.
Fig.5.
Fig.7.
Fig.6.

It seemed possible that the negativity of the infantile ovary depended upon some limiting factor in the internal environment of the animal. In order to investigate this point a limited series of cross-grafting experiments was carried out. The ovary of a non-responsive, four-week old rabbit was grafted under the kidney-capsule of a juvenile rabbit, according to the method of Buyse (1933). Ten days after the graft was made the host animal was started on a five day treatment with unfractionated anterior pituitary extract. The daily dose was the equivalent of 0.5 gram dried sheep pituitary powder, given subcutaneously in two injections. In four cases, where the grafts proved to have taken completely, the grafted ovaries showed no sign of pituitary stimulation and were histologically comparable with either the grafted ovary recovered from an uninjected host animal or with the infantile ovary of the normal six-week old rabbit. The host animal's ovary, however, presented the usual hypophyseal response. Such findings lead one to believe that the internal environment is not to be considered the critical factor involved in rendering the ovary subject to hypophyseal stimulation. It seems, on the contrary, that the negativity of the infantile gonad is inherent within the ovary.

Response of juvenile rabbits to purified follicular-stimulating, and unfractionated pituitary extracts. A series of twenty-five juvenile chinchilla rabbits was given daily subcutaneous injections of pituitary extracts prepared according to the methods described by Fevold, et al. (1933). The daily dose was the equivalent of 0.5 gram dried sheep pituitary powder. Sixteen animals were given the purified follicular-stimulating preparation and nine were treated with an unfractionated extract containing both follicle-stimulating and luteinizing hormones. Injections were continued for periods ranging from five to twenty days. Where macroscopic observations seemed adequate, periodic laparotomies were performed. In some cases, a single ovary was removed after a short period of treatment for histological comparison with the opposite ovary which was obtained after a longer injection period (table 2).

Fig. 1. Infantile rabbit ovary.

Fig. 2. Juvenile rabbit ovary; untreated.

Fig. 3. Juvenile rabbit ovary after five days' treatment with follicular-stimulating extract.

Fig. 4. Juvenile rabbit ovary after 12 days' treatment with follicular-stimulating extract.

Fig. 5. Refractory juvenile rabbit ovary after twenty days' treatment with follicular-stimulating extract.

Fig. 6. Juvenile rabbit ovary after five days' treatment with an unfractionated extract.

Fig. 7. Juvenile rabbit ovary after 15 days' treatment with an unfractionated extract.

After the first five days of treatment with the follicle-stimulating extract the juvenile ovary is enlarged about five-fold (fig. 3) and is constituted almost exclusively of a mass of enormous, translucent, cystic follicles. The interstitial tissue is highly compressed and the number of primordial follicles is greatly reduced. The granulosa cells lining these follicular cysts show all degrees of cytolytic degeneration and in some cases have been practically obliterated. No ovulation has occurred and no traces of luteinization are to be observed.

After about ten or twelve days of continued treatment, the follicular cysts become smaller and the gross dimensions of the gonad are proportionately reduced (fig. 4). If injections are continued for fifteen to sixteen days (fig. 5), the ovary gradually recedes to practically its original gross dimensions. Very few normal appearing follicles remain, and these are comparatively small. There are present, however, numerous connective tissue bodies throughout the ovary. A careful study of the histogenesis of these fibrous masses has shown them to be the remains of the theca walls of a collapsed follicle. Frequently there is enclosed within these bodies a core of peculiarly hyalinized tissue whose origin is not entirely clear. It is nevertheless apparent that a generalized follicular collapse throughout the ovary has produced these fibrous bodies in large numbers. It is to be emphasized that the gradual involution of these cystic follicles occurs without intermediate luteinization and despite continued injection at a uniform daily dose. The ovary seems to have lost its capacity to respond to the continued stimulation afforded it. We have consequently termed such an ovary "a refractory ovary."

When an unfractionated pituitary extract containing both follicular stimulating and luteinizing hormones is injected for graded periods of time, an entirely different series of responses is obtained. After five days' treatment (0.5 gram equivalent daily) the ovary is enlarged about five-fold and is transformed into a mass of corpora lutea (fig. 6). After fifteen to twenty days of treatment, most of these lutein bodies have disintegrated (fig. 7), and the ovary contains enormous masses of tissue which cannot be differentiated from the interstitial tissue of the adult gonad. The remaining lutein cells show extensive vacuolization. The primordial follicles are almost entirely lacking and only a few moribund ova remain. The ovary has receded to practically its original gross dimensions.

It seemed of interest to ascertain whether or not an ovary once rendered refractory can recover its responsiveness to hypophyseal stimulation if allowed to remain untreated for a considerable period of time. Eight rabbits whose ovaries had been rendered refractory by extended pituitary administration were retained for periods ranging from eight to twelve weeks. They showed a normal increase in body weight during this period. Nevertheless, laparotomy at the end of this time revealed that the ovaries

had not undergone the development characteristic of the 5th and 6th month of life. The same pituitary treatment previously employed in each case was repeated and the ovaries in all cases remained entirely unaffected. It thus becomes apparent that the refractory ovary of the juvenile rabbit remains negative to pituitary stimulation for at least two or three months.

Desensitization to pituitary stimulation. We wished to determine whether or not a refractory condition in the ovary must be preceded by a maximal ovarian response. In these experiments two litters of five and one litter of four animals were employed. Twelve of these animals were given follicle-stimulating extract in equivalent of 0.05 gram dried sheep pituitary twice daily for ten days. Laparotomy on the 6th and 11th days after the beginning of the injections revealed that this amount had had no macroscopic affect upon the ovary, and in two cases one ovary was removed for microscopic study. At this point all fourteen animals in this group were started on a dosage of follicle-stimulating extract ample to elicit an extreme ovarian response, namely, 0.25 gram equivalent twice daily for five days. At autopsy the two animals which had not been previously treated with the sub-threshold dose showed the usual ovarian enlargement and follicular development already described. The remaining animals showed no appreciable gross or histological evidence of hypophyseal stimulation. The preliminary injection of the sub-threshold dose seemed to have rendered them insensitive to the hormone subsequently administered.

The effect of brief treatment with luteinizing powder. The effects of brief treatment with the "luteinizing powder" were also studied (table 3). In nineteen out of twenty-one cases the subcutaneous administration of this material in aqueous suspension resulted in the formation of numerous corpora lutea in the ovaries of the juvenile rabbit. Histological study showed that these corpora were commonly derived from the more advanced, vesicular follicles to be found in the ovary of the normal twelve-week old rabbit. In some cases, in addition to the formation of these corpora, there occurred a slight enlargement and mild follicular stimulation throughout the ovary, indicating that the preparation had not been entirely freed of follicle-stimulating hormone. Indeed, it is in these cases that the most extensive luteinization is to be obtained. We have consequently found it most feasible in testing luteinizing preparations to first condition the ovary by means of a mild follicle-stimulating treatment.

Successive injection of follicle-stimulator and luteinizer. The results obtained from successive treatment with the follicle-stimulating and luteinizing fractions are of extreme interest. Thirty-one juvenile rabbits from 12 to 14 weeks of age and weighing from 1300 to 1500 grams were treated in the following manner. They were first given a moderate

follicle-stimulating treatment over a period of three days, the total dose being the equivalent of 1 gram dried sheep pituitary tissue. Laparotomy on the day following showed in most cases moderately enlarged ovaries made up of numerous large, clear follicles. Nevertheless, individual variations in the degree of response were observed, but definite follicular development obtained in all cases. Immediately following this laparotomy administration of the luteinizing fraction was begun. The equivalent of

TABLE 3

*Juvenile rabbits given luteinizing powder**

RABBIT NUMBER	DOSE (IN TISSUE EQUIVALENT)	RESPONSE
	grams	
17	2	**
18	2	**
20	2	**
20A	2	**
28	2	**
28A	2	**
45	0.5	**
49	1	**
51	1	**
54	1	**
66	1	**
67	1	**
69	1	**
70	1	**
71	1	**
80	1	No effect
83	1	**
84	1	**
89	1	**
90	1	**
79	1	No effect

* This material was injected in aqueous suspension, subcutaneously, over five days. Observations made 24 hours after last injection. All rabbits ranged between 1200 and 1500 grams and from 12 to 14 weeks of age.

** Between four and seven well-formed corpora lutea in each ovary. Ovaries show very slight enlargement and in some cases mild follicular stimulation.

1 gram of dried sheep pituitary was injected in six equal parts over a period of three days. Autopsies were performed about 24 hours after the last injection. In 14 of the 31 animals so treated ovulation had occurred. The number of ovulation points ranged from 4 to 10 for each ovary and ovulation was definitely confirmed by the recovery of tubal ova. In addition to the ovulation points these ovaries showed numerous freshly formed corpora lutea and an occasional unaffected follicle. The ovaries

of twelve of the animals in this series also showed extensive luteinization, without any sign of ovulation. Five showed no noticeable response to the luteinizing dose administered, retaining the original condition produced by the follicle-stimulating extract. The basis for this individual variation in response to the luteinizing hormone is as yet obscure.

Associated uterine changes. Associated with these ovarian reactions there occur extensive uterine changes. Cystic ovaries (fig. 3) are invariably accompanied by an enlarged hyperemic, oestrous uterus. Associated with the collapse of the follicular cysts, the oestrous picture in the uterus becomes greatly reduced. These changes may be regarded as an index of the theelin output of the ovary in both the cystic and refractory conditions.

Similarly, an extensively luteinized ovary brings about a progestational modification of the endometrium entirely comparable to that observed in the pseudopregnant adult rabbit. Nevertheless, an ovary which has been subjected to more extended application of an unfractionated pituitary extract is unable to maintain this pseudopregnant picture in the uterus. Under these conditions the uterus recedes to a moderate oestrous condition. It is doubtful whether this uterine reaction is an adequate measure of the corporin output of a refractory luteinized ovary, since it is impossible even with corpus luteum extracts to maintain a progestational condition in the uterus for more than about ten days (Leonard and Hisaw, 1930).

Discussion. It has been shown by Corey (1928) that the ovary of the albino rat will not respond to pituitary hormones until the 11th day of life. Hargitt (1930) in his thorough study of the post-natal differentiation of the gonads of the rat, has shown that the 11th day of life marks the first occurrence of incomplete follicular antra. We have previously seen that in the rabbit, also, the time at which antra folliculi first occur coincides with the earliest pituitary response. Whether or not this coincidence is indicative of a cause and effect relationship between these two phenomena is entirely problematical. Nevertheless, it becomes clear that in the rabbit as well as in the rat an ovary which is free of follicular antra shows no follicular development after treatment with the recognized pituitary hormones.

In addition, the graft experiments described above indicate that merely transferring such an infantile ovary to the internal environment of an older animal is not sufficient to render it responsive to pituitary stimulation. It seems rather that the ovary itself must first undergo certain developmental processes. The chief morphological changes to be observed during this prepubertal development of the ovary are: 1, a gradual increase in size of the ovary as a whole; 2, reduction in the number of oocytes; and 3, the formation of antra-containing follicles. The present experiments do not afford conclusive evidence that the factors effecting these changes

are extra-hypophyseal, still it is of importance to note that with the pituitary preparations here employed no such prepubertal ovarian development could be brought about. It is possible that the time involved in ovarian differentiation is an essential and not merely an incidental factor in this process.

Cushing (1932) in his discussion of the condition of the ovaries in cases of pituitary basophilism, states that he is at a loss to account for the fact that in this extreme hyperpituitary condition atrophic ovaries are usually found. It is highly probable that the refractory condition described here is the basis for this apparent discrepancy. In all events, it is obvious that the momentary condition of a gonad is not always an adequate indication of the degree of hypophyseal stimulation to which the ovary is being subjected.

Zondek (1931) showed that in post-climacteric individuals the urine contains considerable quantities of a pituitary-like substance termed Prolan A. It is thus apparent that the termination of ovarian activity is not necessarily associated with the cessation of hypophyseal secretion. On the contrary, a certain degree of refractoriness on the part of the ovary seems to be a distinct factor. The refractory condition which we have here produced constitutes substantial experimental evidence favoring this point of view, and may help account for the progress of senility despite continued hypophyseal secretion.

The deleterious effects of continued treatment with an unfractionated pituitary extract bear a striking resemblance to the picture produced by x-ray treatment of the rodent ovary (Parkes, 1926–1927; Zondek, 1927). In both instances there obtains a practically complete destruction of ova and Graafian follicles and an excessive abundance of interstitial tissue. The uteri associated with this ovarian condition in the rabbit show evidence of mild theelin stimulation. Similarly, Parkes (1926) has shown that the ovary of the mouse brought to a comparable state by x-ray irradiation continues to elaborate effective quantities of theelin. In addition, Zondek (1931) states that the previously x-rayed ovary will give no response to pituitary administration. Our experimental animals likewise proved refractory to further hypophyseal stimulation. It seems, then, that both histologically and functionally the ovarian condition resulting from continued administration of an unfractionated pituitary extract is somewhat comparable to that which follows x-ray irradiation.

We have pointed out above that the preliminary injection of a subthreshold dose of follicle-stimulating extract desensitizes the recipient to even maximal doses subsequently administered. Gross histological methods reveal no ovarian change which can account for this reaction. Nevertheless, it is known that the ovaries of immature pituitary-treated animals may even produce effective quantities of theelin before they show

any histological signs of hypophyseal response (Fels, 1930). One can consequently not be certain from ordinary histological data that some reaction on the part of the ovary itself is not involved in the desensitization process. Perhaps cytological study of the granulosa cells of the previously responsive follicles would bring us to a better concept of the significance of this desensitization phenomenon.

In the present work ovulation was induced only in those animals which received a follicle-stimulating treatment followed by a luteinizing treatment. It occurred in 45 per cent of the animals in this group. We are unable to account for this individual variation to a treatment which was kept constant as far as possible. Nevertheless the occurrence of ovulation only after a combined treatment with follicle-stimulating and luteinizing preparations throws some light upon the physiology of ovulation, indicating that this process may depend on a synergistic balance between the two gonadotropic hormones, and cannot be brought about in the immature animal by the use of either factor alone. The present evidence, however, by no means warrants any conclusion on this point.

The present work also has a bearing upon the problem of the duality of the gonadotropic hormones secreted by the pituitary. One of the most critical objections to the two hormone theory is based upon the fact that even the most purified follicle-stimulating extracts hitherto prepared would induce luteinization, if injected at high dosages or for extended periods of time (Engle, 1932). Such objections were raised particularly with regard to the rat and mouse, and on the basis of data obtained with relatively unpurified tissue extracts In the present work with the rabbit we have seen that it is possible by the use of a purified extract to carry a Graafian follicle through a cystic phase on down to its complete collapse without intermediate luteinization, despite continued application of excessive hormone dosages It has been shown further that extensive luteinization may be produced in the immature ovary either by the administration of comparable doses of an unfractionated extract or by the successive injection of follicle-stimulating and luteinizing preparations. It is thus apparent that an additional and distinct stimulus is required for luteinization.

SUMMARY

1. The ovaries of twelve four-week-old infantile chinchilla rabbits weighing between 500 and 700 grams remained unmodified after treatment with either follicle-stimulating or unfractionated pituitary extracts in doses which, in juvenile animals between 12 and 14 weeks of age and weighing between 1300 and 1500 grams, invariably elicit a maximal ovarian response.

2. Histological comparison of the infantile and juvenile type of ovary shows the latter to be much more highly differentiated particularly with regard to the formation of antra-containing follicles.

3. The infantile ovary even when grafted under the kidney-capsule of a juvenile host proved unresponsive to pituitary stimulation with the extracts here employed (4 cases).

4. Sixteen juvenile rabbits were given daily subcutaneous injections of a purified follicle-stimulating extract for periods ranging from 5 to 20 days. After five days the ovaries were enlarged about five-fold and were made up of large cystic follicles with relatively little interstitial tissue. After about ten days of continuous treatment the ovaries gradually decreased in size and by the fifteenth day of treatment had receded to approximately their original gross dimensions. This rapid involution was associated with a generalized follicular collapse throughout the ovary and proceeded without intermediate luteinization of the follicles concerned.

5. Nine juvenile rabbits were given daily subcutaneous injections of an unfractionated pituitary extract for periods varying from 5 to 20 days. After five days the ovaries increased in size about five-fold and showed extensive luteinization. After about ten days the ovaries gradually decreased in size and by the fifteenth day had reached practically their original gross dimensions. This involution was associated with a disintegration of the experimentally produced corpora lutea and an excessive deposition of interstitial tissue.

6. Eight rabbits whose ovaries had been rendered refractory to further pituitary stimulation by prolonged treatment were allowed to remain untreated for two to three months. At the end of this time a second treatment showed that the ovaries of none of these animals had recovered their responsiveness to hypophyseal stimulation.

7. Twenty-one juvenile rabbits were given subcutaneous injections of a luteinizing extract for five days. Nineteen of these animals showed very slight ovarian enlargement and several corpora lutea. The ovaries of the remaining two animals were unaffected.

8. Successive treatment with follicle-stimulating and luteinizing extracts in thirty-one juvenile rabbits elicited ovarian enlargement and extensive luteinization with ovulation in 14, ovarian enlargement and extensive luteinization without ovulation in 12, and only follicular development and ovarian enlargement in five.

9. Preliminary treatment with a sub-threshold dose of follicle-stimulating extract rendered fourteen juvenile rabbits insensitive to even maximal doses subsequently administered.

10. Discussion is given concerning the bearing which the present work has upon *a*, the problem of the prepubertal conditioning of the ovary; *b*, the occurrence of gonadal hypoplasia in clinical cases of hyperpituitarism; *c*, the progress of senility despite the continuation of hypophyseal secretion; *d*, the physiology of ovulation; *e*, the question of the duality of the gonadotropic pituitary hormones.

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THE EFFECT OF ARSENITE ON MEDULLATED NERVE

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Arsenious acid and other trivalent arsenic compounds are known to exert a strong inhibitory action on the metabolic processes of a variety of animal tissues (Onaka, 1911; Dresel, 1926; Szent Gyorgi, 1930; Voegtlin, Rosenthal and Johnson, 1931). The degree of this inhibition, 60 to 80 per cent in millimolar solutions, indicates clearly that arsenic produces its effect by poisoning one or more of the catalytic systems in the cell. The literature, to be discussed in a paper to follow, reveals little concordance of views regarding the probable mechanism of this effect.

Preliminary experiments with frog sciatic nerve showed that arsenic not only causes a high degree of inhibition of respiration but also abolishes irritability in such low concentrations as to obviate any explanation but one based on a catalytic mechanism.[1] The phenomena are undoubtedly produced by a disturbance of certain fundamental processes, such as oxygen or hydrogen activation or possibly the transport of these activated substances by carrying mechanisms, such as the cytochrome or sulphydryl systems. Since data from the relatively few studies on nerve oxidases, dehydrases, transport systems, etc., have been procured from mammalian tissue, little information is available concerning these fundamental processes of activation and of transport in cold blooded nerve. Nevertheless, since cold-blooded nerve is less abnormal than excised mammalian nerve, and since the electrophysiological properties have been worked out most successfully in cold blooded nerve, it is desirable that the catalytic mechanisms be studied for this tissue also. Hence, we approached the problem of elucidating the mechanism of the arsenic effect, not only for its own sake, but also because it necessitated an investigation of the various oxidation mechanisms in frog nerve as a base line upon which to work.

In the present paper we describe experiments on the effect of arsenic on the respiration and action potential. A closer analysis of the nature of the catalytic mechanisms involved in these arsenic effects will be reported in a paper to follow.

[1] These preliminary experiments were performed by one of us (F. O. S.) and Mr. S. Lake in the summer of 1932.

METHODS. A stock solution of arsenious acid was prepared and standardized according to the method described by Treadwell and Hall (1919). This stock solution was usually from 0.1 to 0.3 M and was made up fresh every week or so. The acid was brought to the desired concentration by dilution with Ringer solution containing 10 per cent by volume of isotonic phosphate buffer, pH 7.6–7.8. In concentrations of arsenite up to 0.01 M, it was found that this strength of buffer sufficed to maintain the pH within the desired range of 7.6–7.8 as measured with the glass electrode. In the few instances in which solutions more concentrated than 0.01 M were used the acid was either neutralized with NaOH before adding the phosphate Ringer or the proportion of isotonic buffer was increased to 50 per cent by volume instead of 10 per cent.

Respiration was measured both in differential manometers (Warburg technique) and in differential volumeters (Thunberg technique, as modified by Fenn). The two methods gave results which agreed in all respects. The inhibiting effect of arsenic was determined in two ways. In the first method the nerves were placed in the appropriate arsenic solution at the beginning of the experiment and the rate of respiration compared with that of symmetrical nerves in phosphate Ringer as a control. In the second method the nerves were placed in a small quantity of phosphate Ringer in vessels provided with a side arm. In the side arm was placed 0.2 cc. arsenite in buffer. After obtaining the resting rate in phosphate Ringer, the arsenite was tipped and the respiration again followed. The latter method requires no control nerves and also permits one to follow the rate from the moment of exposure to arsenite.

Action potentials were determined oscillographically. Nerves were dissected in symmetric pairs and the spike heights followed for a preliminary period in phosphate Ringer. Then after this preliminary run, the experimental nerve was placed in arsenite, the control nerve remaining in phosphate Ringer. Except for the time required to withdraw the nerves from the arsenite or phosphate Ringer to test the action potential, they were kept in beakers or in Warburg vessels submerged in a constant temperature thermostat. It was found that if the adhering fluid be removed by gently drawing the nerve over a dry glass surface, the action potentials of the control nerves checked from one determination to the next usually within 5 to 10 per cent. Ringer solution containing 10 per cent isotonic phosphate buffer, while not an ideal milieu for surviving nerve, causes the action potential to drift down only very slowly (ca. 50 per cent in fifteen or twenty hours); in view of the fairly prompt extinction of the arsenic treated nerves, this serves sufficiently well as a control base line. This is true when the temperature is kept at about 20–22°C.; at room temperature (26–30°C.) even the control nerves show considerable downward drift in action potential.

EXPERIMENTAL RESULTS. 1. *The effect of arsenite on nerve respiration.* In conformation of the results of other workers on mammalian tissues, we find that arsenite produces inhibitions of the order 60 to 80 per cent in concentrations of 0.001 M or higher. The data are summarised in table 1, and figure 1 is a graphic presentation of the data of a typical experiment (no. 57). Even with 0.0001 M the inhibition is still considerable (ca.

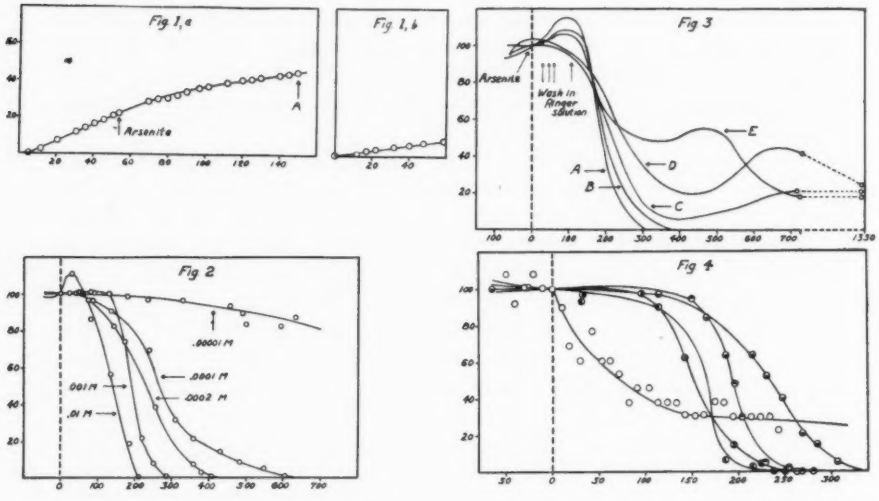

Fig. 1. Effect of arsenite on nerve respiration. Ordinates, oxygen consumption in cubic millimeters per gram; abscissae, time in minutes. Arsenite was tipped onto the nerves at arrow, the final concentration being 0.0005 M. At *A*, nerves were removed from vessels and washed for 135 minutes in Ringer solution. Respiration after washing is shown in figure 1b.

Fig. 2. Effect of arsenite on action potential. Ordinates, action potentials in per cent of original value; abscissae, time in minutes. Nerves were transferred to arsenite solution at zero time.

Fig. 3. Irreversibility of arsenite effect. All nerves were placed in 0.0011 M arsenite solution at zero time. After 30 minutes, nerve *E* was removed from arsenite and placed in Ringer solution. Nerves *D*, *C*, and *B* were similarly returned to Ringer solution after 45, 60, and 108 minutes, respectively. Nerve *A* remained in arsenite continuously. The points at the extreme right of the group are included to show that no further recovery occurred even after 1350 minutes of washing in Ringer solution. Coördinates as in figure 2.

Fig. 4. Induction period in arsenite effect on action potential. Open circles indicate *rate* of respiration of group of four nerves; partly closed circles indicate action potentials of each nerve of the comparable group measured separately. Arsenite added at zero time to make final concentration of 0.001 M. Ordinates, action potentials and *rates* of respiration in per cent of original value in Ringer solution; abscissae, time in minutes.

40 per cent), and slight but definite inhibitions can be demonstrated with concentrations as low as 0.00002 M.

Contrary to some of the earlier work (Onaka, 1911) we have observed little reversibility of arsenic inhibition of respiration. The reversibility of the poisoning was tested by washing the arsenic treated nerves for one or two hours after the preliminary run had shown that the maximum inhibition had been achieved; the respiratory rate after washing was then compared with the original rate before treatment with arsenic (see fig. 1). Partial recovery was observed in only two instances in a series of eight experiments.

That the arsenic inhibition is not due to an ion imbalance caused, perhaps, by calcium demobilization is shown by the fact that inhibition may be obtained with very high dilutions of the arsenite. Furthermore cal-

TABLE I

The effect of arsenite on respiration of nerve

EXPERIMENT NUMBER	TEMPERATURE	TYPE OF RESPIROMETER	CONCENTRATION OF ARSENITE	INHIBITION
series A	*°C.*		*M.*	*per cent*
43	17.6	Volumeter	0.0252	83
32	15.1	Manometer	0.0152	58
14	21.5	Manometer	0.0047	74
28	20.0	Volumeter	0.0019	75
57	22.0	Manometer	0.0010	64
50	18.0	Volumeter	0.0004	57*
17	21.5	Manometer	0.00018	33
52	20.9	Volumeter	0.00009	48*
39	15.2	Manometer	0.000037	20
54	21.2	Volumeter	0.000018	16*

* Nerve sheaths split.

cium demobilization produced by citrates, tartrates, etc., instead of inhibiting nerve respiration, actually stimulates it (Chang, Gerard and Shaffer, 1932).

The arsenite inhibition of nerve respiration is in many respects similar to cyanide inhibition. Comparing the data of Schmitt and Schmitt (1930) with those contained in table 1, we see that while cyanide produces a somewhat greater inhibition at high concentrations, arsenite seems to be somewhat more effective in lower concentrations. The actual degree of inhibition with low concentration of cyanide, however, is uncertain because of the escaping tendency of HCN. This similarity between the cyanide and arsenite inhibition of respiration was noted by Onaka (1911).

2. *The effect of arsenite on the action potential.* Like cyanide, arsenite abolishes irritability of frog sciatics in low concentrations. The data are

summarized in table 2. In general the shape of the spike height-time curve for arsenite resembles that for cyanide (see Schmitt and Schmitt, 1930). Figure 2, which is a graphic summary of the data from experiments 24, 75, 62, 71 and 73, illustrates this point. There is an initial period, greater for arsenite than for a similar concentration of cyanide, in which the spike height remains unaffected. This is followed by a rapid decline which, after a period of tailing out, ends in complete extinction of the action potential.

Arsenite action differs from that of cyanide, narcotics or ion blocks in that its effects do not appear to be reversible. Once the action potential

TABLE 2

The effect of arsenite on the action potential

EXPERIMENT NUMBER	TEMPERATURE	METHOD*	CONDITION†	CONCENTRATION	EXTINCTION TIME
series B	°C.			*M.*	*minutes*
91	21	M	B	0.10	156
24	30	B	m	0.011	210
55	20	M	m	0.0025	225
53	20	M	m	0.0012	237‡
61	20	M	m	0.0010	296
75	21	B	d	0.0010	285
52	20	M	m	0.00070	240‡
54	20	M	m	0.00037	319‡
62	20	M	m	0.00020	411
71	20	B	d	0.00010	604
65	20	m	m	0.00001	Unaffected in 24 hr.
73	22	B	d	0.00001	Unaffected in 24 hr.

* B signifies that the nerves were kept in beakers in the thermostat between readings; m signifies that they were kept in Warburg vessels, attached to manometers and shaking in the thermostat.

† M signifies that the nerves were burnt to make the action potentials monophasic; d means that the nerves were not burnt and the action potentials were therefore diphasic.

‡ Split sheath preparations.

has been reduced to extinction, no matter how dilute the arsenite, washing in Ringer solution fails to restore irritability in the slightest degree; this was the invariable result in every experiment. Indeed, one may go farther and say that unless the nerve is withdrawn from the arsenite before the action potential begins to fall (end of induction period), subsequent washing in Ringer solution does not prevent the eventual extinction of the action potential. Figure 3 illustrates this point. After 30, 45 and 60 minutes' exposure, an increasing number of the fibers have been irreversibly affected by the arsenic and the final action potential height after washing is an index of the number of fibers which have not been so af-

fected. After exposures longer than 60 minutes, all fibers have been irreversibly affected and irritability is abolished in spite of the fact that the nerve was removed from the arsenite solution and washed in Ringer solution before any deleterious effect on the action potential could be detected.

A few experiments were performed to test the possibility that the arsenite effect on irritability might be due to demobilization of calcium. It was found that whereas millimolar arsenite caused the extinction of the action potential in about four hours, millimolar solutions of citric or tartaric acids had no significant effect on the spike height for the entire duration of the experiment (eight hours).

3. The arsenite "induction period." It will be observed from figure 1 and figure 2 that the arsenite effect (maximum inhibition of respiration and extinction of the action potential) does not manifest itself for a period of one to three hours or more after exposure of the nerves to arsenic. It was at first thought that this delay was due to slowness of diffusion caused by the epineural sheath (see Feng and Gerard, 1930). Against this view, it was found that a similar though perhaps slightly shorter "induction period" was observed both for the time of onset of maximal inhibition of respiration and for extinction of the action potential when the nerve sheaths were split. Inspection of the figures published by Voegtlin, Rosenthal and Johnson (1931) reveals a similar induction period even with suspensions of macerated testis material where the diffusion factor must be negligible. Furthermore, bull-frog motor roots which are not enclosed in a tenuous sheath likewise show an induction period (with regard to the extinction of the action potential) of the same order of duration as that of frog sciatics.

It is of considerable theoretical importance to determine how much of this induction period is due to diffusion factors and how much may be attributed to an actual latency of action. If the latter factor can be demonstrated to be genuine, then arsenite probably causes depression of irritability by action on a system other than the immediate substrate for conduction. Its action would be comparable to that of HCN, CO, and other asphyxiating agents.

The solution of this problem was achieved in the manner illustrated in figure 4. Sciatics from four frogs were dissected and divided into two comparable and symmetrical groups. One group of four nerves was used for respiration determinations, the other group for measurements of action potential. The respirometers were small Warburg vessels provided with an inset for alkali and a side arm. The nerves were placed in 0.4 cc. phosphate Ringer with 0.2 cc. of 0.003 M arsenite in the side arm and 0.2 cc. alkali in the inset. A similar vessel containing the same fluids but no nerves served as a control and was placed on the other arm of the differential manometer. During these operations, the nerves of the other

groups were placed, one each, in a "Kästchen" vessel on a simple manometer and were shaken in the same thermostat with the differential manometer. Every ten minutes or so, one of the Kästchen vessels was removed from its simple manometer on the shaker and the nerve contained therein examined oscillographically and the spike potential recorded. In this manner we were able to record simultaneously the respiration and action potentials of comparable nerves similarly treated. After sufficient time had elapsed to give linear respiration and a steady action potential, arsenic was tipped from the side arm of the respirometer vessels and at the same time the phosphate Ringer solution bathing the individual nerves in each "Kästchen" vessel was changed to Ringer solution containing 0.001 M arsenite. It will be seen that whereas the respiration rate began to fall within a few minutes, the action potential remained unaffected for a considerable time. Indeed, no change in the action potential height was observed until the respiration had almost reached a steady rate corresponding to the maximal degree of inhibition. These results show beyond peradventure that the time lag in the arsenic effect on the action potential is due to a factor other than diffusion. Whether there is significance in the fact that the action potential decline begins at the time the respiration has just come to its maximal inhibition, we are unprepared to say. That there is an induction period also in the inhibition of respiration seems to follow from the fact that there is little difference in the shape of the curves whether the nerve sheaths have been split or not. Further discussion of the significance of this induction period is reserved for a paper to follow.

SUMMARY

1. Arsenite inhibits nerve respiration in very low concentrations (about 40 per cent in 0.0001 M solutions). This inhibition is only partially reversible at low concentrations and is irreversible in concentrations as high as millimolar.

2. Arsenite irreversibly abolishes the action potential in solutions which are 0.0001 M or stronger. These effects do not manifest themselves immediately upon exposure to arsenite but only after an "induction period" of one to three hours. This "induction period" cannot be accounted for by diffusion factors.

3. Reasons are given for supposing that these arsenite effects are not produced by ion imbalance such as by demobilization of calcium, but are produced by an anticatalytic effect presumably upon oxidative systems.

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THE HYPOPHYSEAL-LIKE QUALITIES OF THE GONADOTROPIC PRINCIPLE FOUND IN THE URINE OF CERTAIN INDIVIDUALS[1]

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It was first demonstrated by Zondek (1930) that the urine of ovariectomized women, women past the menopause, and individuals with certain types of genital tumors contained a gonadotropic hormone which was different from that found in the urine of pregnant women. Extracts of these urines usually stimulated only follicular growth in sexually immature rodents in a 100-hour period, though occasionally corpora lutea were formed. A differential action between this urine and that of pregnancy has also been revealed by the rabbit ovulation test (Leonard, 1931). In an article on migraine (Riley, Brickner and Kurzrok, 1933), Kurzrok shows in his illustrations an ovarian response in which follicular growth is predominant after injection of urinary extracts from certain of their patients into normal immature mice. Hamburger (1933) has also shown differences between the action of the gonadotropic principle in urine from pregnant and from castrated women when injected into immature mice and rats. The P.U. matured but few follicles, whereas the castrate urine induced growth in many follicles. The infantile rat was 5 times as sensitive as the mouse to P.U., whereas the mouse was 2 times as sensitive as the rat to castrate urine.

The difference shown by all these tests in normal animals is quantitative and not qualitative. It seemed to us that hypophysectomized rats might show a qualitative difference between the two types of urine for the injection of P.U. extracts in them does not induce any follicular growth (Collip et al., 1933; Leonard and Smith, 1933). The use of hypophysectomized rats has revealed a more striking qualitative difference between the action of the gonadotropic principle in the follicle-stimulating urine

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[2] National Research Fellow in the Biological Sciences.

(F.-S.U.) extract than we had anticipated.[3] The differences and similarities between the ovarian response elicited by the F.-S.U. extract and A.P. implants in this experimental form are also of considerable interest.

A second test applied to the F.-S.U. extracts was the "augmentation" test. It has been shown by Leonard (1932) that the increase in the ovarian weights beyond the calculated amount, which occurs when P.U. extracts are given concurrently with A.P. extracts in rats, is not due to the activation of a growth hormone in the latter as first claimed by Evans et al. (1932) but involves an interaction of the contained gonadotropic substances. If the F.-S.U. extract contains a principle physiologically similar to the A.P. gonadotropic hormones, especially if it is rich in a follicle-stimulating substance, then the concurrent injection of the P.U. and F.-S.U. extracts should give an ovarian increase greater than the calculated combined increases of the injected individual substances.[4]

The results given in this paper will show 1, a striking qualitative difference between the ovarian responses evoked in hypophysectomized female rats by the injection of F.-S.U. extract and of P.U. extract, and 2, an "augmentation" effect when these two substances are injected concurrently in normal immature female rats.

The extract. Pooled morning samples of urine (1700 cc.) known to contain a gonadotropic hormone were obtained from an individual (Mrs. H.) who was past the menopause and was subject to attacks of migraine. The urine was acidified with glacial acetic acid and 4 volumes of 95 per cent alcohol added. The precipitate was washed several times with ether, dried, and stored in crude powder form till used.

A total dosage of the F.-S.U. extract equivalent to 10 cc. of urine, when injected over a period of 3 days (1 injection each day) tripled the size of the ovaries of sexually immature mice (22 days old) in a 100-hour period. The ovaries contained only follicles except in 1 of 7 animals in which luteinization had begun. The same treatment in 22-day old immature rats had no detectable effect on the ovaries or uterus. This result is in harmony with the findings of Hamburger but not of Zondek (1931), who states that the rat is more sensitive to Prolan A (F.-S.U.) than to Prolan B

[3] Since the printing of "follicle-stimulating urine," a name which at present appears to characterize most correctly the physiological action of this urine, takes an undue amount of space, we have adopted the first letter of each name as an abbreviation and will refer to it in this paper as F.-S.U. This seems not unfitting, since pregnancy urine is commonly abbreviated to P.U. It seems wise not to use the names of the patented products, Prolan A and Prolan B.

[4] Fevold et al. (1933) report that concurrent injections of P.U. extract and an A.P. follicle-stimulating extract give augmentation, whereas concurrent injections of P.U. and an A.P. luteinizing extract do not give an increase above that which would be expected. The luteinizing extract combined with the follicle-stimulating preparation, however, gives augmentation.

(P.U.). The equivalent of 22 cc. of urine given in 4 daily injections usually increased ovarian weights only slightly in immature rats, although in one animal the weight of the ovaries was increased markedly and the uterus was enlarged. None of the normal immature rats injected with doses of urinary extract from 10 to 40 cc. of urine equivalent, showed evidence of luteinization in 120 hours even though the ovaries in the larger dosages weighed up to 0.045 gram.

Injections in hypophysectomized rats. Three litters of immature, hypophysectomized rats were used in these experiments. A post-operative period elapsed in each case before treatment was begun. One cubic centimeter of the F.-S.U. extract was equivalent to 20 cc. of urine.

Protocol, litter A. Three animals, 32 days old at hypophysectomy, weights 66 to 86 grams. No treatment for 12 days. One animal (G9613) was injected with 1 cc. of the extract daily for 4 days, and autopsied the next day, ovaries, 0.0230 gram, uterus, 0.085 gram. The second animal (W9614) was injected with $\frac{1}{2}$ cc. of the extract daily for 10 days. At autopsy the following day the ovaries weighed 0.0595 gram and the uterus, drained of the fluid which distended it, 0.136 gram. The third animal (GH9615) remained uninjected for 17 days at which time the left ovary was removed, weight 0.0040 gram. This animal was then injected with $\frac{1}{2}$ cc. daily of F.-S.U. extract for 5 days, following which 1 cc. daily was given for 3 more days. At autopsy, the following day, the right ovary weighed 0.0230 gram, the uterus 0.156 gram.

Structural findings. The control (left) ovary of GH9615 showed the atrophy characteristic of hypophysectomized animals (fig. 1). The other ovary, following treatment, and the ovaries of the other two (treated) animals showed numerous large clear follicles of uniform size under the binocular dissecting microscope. No corpora lutea or blood points could be seen. In section, no corpora were found, although in an occasional follicle, in the longer treatments, small localized areas of the follicular wall were seen to be undergoing luteinization. The follicles in the short (4 day) treatments and those of the longer treatments, except for an occasional one in the latter which showed slight luteinization, appeared like large normal follicles just before ovulation. They were strikingly uniform in size. Each contained a normal ovum (fig. 2).[5] None of the follicles had ovulated as shown by a microscopic examination of the ovaries and the tubes.

The effects produced by the injection of the F.-S.U. extract differ strikingly from those of P.U. extract. Not only did it cause growth and

[5] An interesting anomaly was observed in a follicle of one of the ovaries. A large normal follicle was completely enclosed in a second somewhat larger follicle. The thecae of the two were attached at one side, at which attachment no granulosa cells were present. Each of the follicles contained a normal ovum.

maturation of follicles, an effect which is not given by P.U. injections, but the interstitial and theca cell hypertrophy invariably produced by P.U. extract occurred but slightly if at all in the F.-S.U. injected animals.

Figure 2 illustrates a typical section from an ovary of an hypophysectomized rat treated with the F.-S.U. extract. It will be readily seen that the structure is very similar to that of immature rats or mice in which precocious sexual maturity has been induced by A.P. implants. It was in such animals that superovulation was secured (Smith and Engle, 1927).

Since ovulation had not taken place in the F.-S.U. injected operated animals, although the maturation of numerous follicles had been induced by the treatments, we determined to try with another litter a procedure by which one of us had secured ovulation in immature normal rats (Leonard, 1931). This was to inject for a period with a follicle-stimulating substance followed by a single intraperitoneal injection of an extract of pregnancy urine.

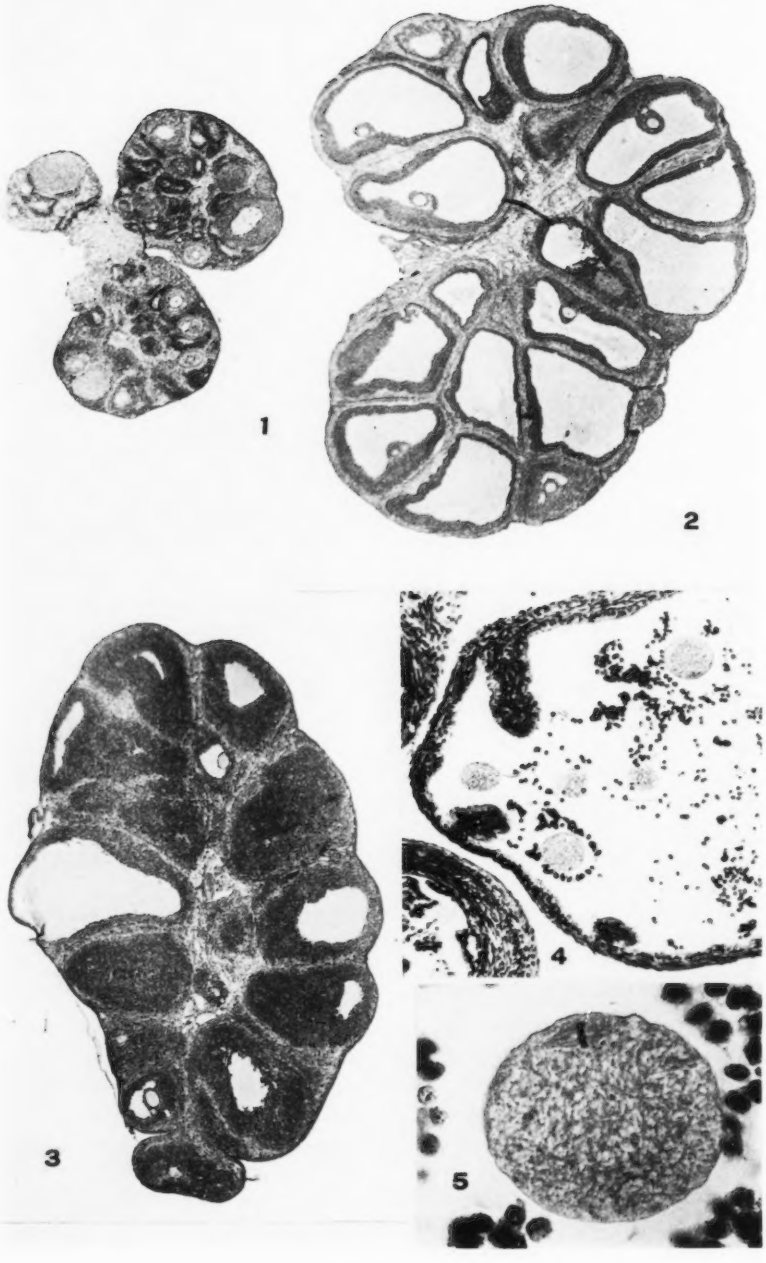
Protocol, litter B. Litter of 4 immature rats, 3 of which were hypophysectomized when 35 days old (64–72 gm.) and 1 was reserved as an unoperated control. No treatment for 14 days. Beginning on the 15th day following the pituitary ablation, 1 cc. of the F.-S.U. extract was injected daily for 5 days in 2 of the operated rats (BH9750, B9753). On the 6th day the left ovary of each animal was removed, the weights being 0.0145 gram and 0.0325 gram respectively. Following the operation 1 cc. of Antuitrin S was injected intraperitoneally into each of the two animals. Twenty-four hours later they were autopsied, and the right ovaries found to weigh 0.0230 gram and 0.0335 gram respectively. The uteri weighed 0.090 gram and 0.107 gram. In each of these ovaries small ruptures suspected to be ovulation points were evident.

At the time that the left ovaries of the two above animals were ablated, the control (untreated) littermate was autopsied. The combined weights of its ovaries were 0.030 gram, weight of uterus, 0.236 gram.

Results. A comparison of the ovarian weights of these animals showed that the ovaries of the two operated rats at the termination of the F.-S.U. extract injections were 4.5 and 10 times as heavy, respectively, as the ovaries of the untreated operated control. In each case the ovaries of these two treated operated rats were heavier than those of the untreated normal control. These results were similar to those of litter A.

The left ovaries of the two treated, operated rats (removed at the end of the F.-S.U. extract injections, but before the intraperitoneal injection of P.U.) showed the same morphological picture as has been described in the injected operated animals of litter A (fig. 2). There were many uniform-sized normal follicles, each of which contained a normal ovum.

The right ovaries of these two rats (removed 24 hours after the intraperitoneal injection of P.U. extract) showed, in section, definite ovulation



Figs. 1 to 5

points. There was one blood follicle. Luteinization was almost entirely absent (fig. 3).

A study of serial sections of the ovarian tubes of the operated animals which had been injected with the F.-S.U. extract followed by P.U., given intraperitoneally, *revealed the presence of 6 eggs in the right tube of one animal and 21 eggs in the right tube of the other animal.* In each of the eggs which were studied serially, a polar spindle was present (fig. 5). The eggs had lost the cumulous cells, however, indicating some delay in polar body formation (figs. 4, 5). Apparently ovulation had taken place immediately following the intraperitoneal injection of the P.U. extract.

Protocol, litter C. Litter of 6 immature rats, all of which were hypophysectomized when 28 days old (50–59 gm.). No treatment for 5 days. F.-S.U. extract (½ cc. daily) was then injected for 5 days in 5 of the animals, at the termination of which one was autopsied, weight of both ovaries 0.0332 gram, of the uterus 0.117 gram. The untreated, operated animal was also autopsied at this time, weight of both ovaries 0.0112 gram, uterus 0.0301 gram. The remaining 4 animals were injected intraperitoneally at this time with 1 cc. of Antuitrin S (100 R.U.). Twenty-four hours later 2 of them were autopsied, weights of both ovaries, 0.0542 gram and 0.0747 gram respectively, and the uteri 0.125 gram and 0.120 gram. Forty-eight hours after the Antuitrin S injection the remaining 2 rats were autopsied, weights of ovaries 0.1281 gram and 0.1127 gram, respectively.

Results. The ovaries of the untreated (operated) rat showed the characteristic atrophy. Those of the animal which was injected with F.-S.U. extract and autopsied at the termination of the treatment showed a pronounced growth of the follicles. In some of the follicles the ova had become detached from the cumulus. None of the tubes of this animal or of those of the rats which had, in addition, been injected with P.U. extract contained ova. All the follicles of the latter contained ova which lay free in the cavity. They had spindles. The granulosa of the follicles in the animals killed 24 hours after the P.U. extract injection had thickened

Fig. 1. The left (control) ovary of an immature rat (GH9615, litter A) removed 17 days after hypophysectomy. No treatment. ×28.

Fig. 2. The right ovary of the above animal removed 8 days later during which time an extract of follicle-stimulating urine had been injected. Total urine-equivalent, 110 cc. ×28.

Fig. 3. The right ovary of an immature hypophysectomized rat (BH9750, litter B). Following a post-operative period of 15 days, during which time no treatment was given, an extract of follicle-stimulating urine was injected for 5 days. The next day the left ovary was removed and 100 R.U. of antuitrin S injected. Autopsy 24 hours later. Twenty-one ova were found in left tube (see figs. 4 and 5). The granulosa which is much thickened does not show luteinization. ×28.

Fig. 4. A group of ova from the right oviduct of the above animal. ×94.

Fig. 5. An enlargement of one of the ova. ×495.

but did not show luteinization (fig. 3). The follicles of the animals killed 48 hours after the P.U. injection uniformly showed luteinization, a large central cavity, however, remaining.

The uteri were enlarged and in some cases distended with fluid in all the operated animals of the 3 series which were injected with F.-S.U. extract, showing that the injections had stimulated the ovaries to produce oestrin. The production of oestrin is also evidenced by the vaginal changes. Since the rats were hypophysectomized when immature, the vagina had remained closed throughout the pre-treatment period. The vagina opened on the 4th or 5th day from the beginning of treatment with a pro-oestrous type of smear which changed to a cornified type.

Serial sections of the pituitary capsule of each of the hypophysectomized animals were carefully examined for fragments of A.P. None were found.

TABLE 1

Augmentation with P.U. extract and follicle-stimulating urinary extract (F.-S.U.) in normal immature female rats

(The animals in each line were littermates of uniform size)

TREATMENT			AVERAGE OVARIAN WEIGHT (MGM.)			PER CENT AUGMENTATION
P.U. ext. R.U.	F.-S.U. ext. Conc. 1:20 cc.	Combined P.U. + F.-S.U.	P.U. treated	F.-S.U. treated	Combined treated	
	cc.	*cc.*				
10	1	10 + 1	(1) 27.0	(1) 18.0	(2) 46.0	107
20	1	20 + 1	(1) 47.5	(1) 16.0	(2) 78.3	89
100	1	100 + 1	(2) 39.0	(1) 12.5	(2) 108.0	288
100	1	100 + 1	(2) 37.2	(2) 24.8	(2) 89.0	130
50	1.5	50 + 1.5	(1) 31.7	(1) 16.2	(2) 69.4	204

* Number of animals injected is shown in ( ).

"Augmentation" in normal immature rats. When a P.U. extract is injected concurrently with a gonadotropic hypophyseal extract containing a follicle-stimulating substance in normal immature rats, the increase in the weights of the ovaries is greater than that which would be predicted by adding the increases produced by each of the extracts injected individually (Leonard, 1932). This has been confirmed by Fevold, Hisaw, Hellbaum and Hertz (1933) and by Evans, Simpson, and Austin (1933). Since "augmentation" had been secured only by combined injections of an hypophyseal extract and pregnancy urine, it seemed possible by injecting the F.-S.U. and P.U. extracts, concurrently, to secure evidence as to whether the substance in the follicle-stimulating urine was similar to the pituitary gonadotropic principle reacting with pregnancy urine.

As will be seen from the data (table 1) "augmentation" resulted, the increases from the combined injections varying from 91 to 288 per cent

over the calculated. This result indicates that the follicle-stimulating urine secreted by this individual (Mrs. H.) contains a substance which has definite hypophyseal characteristics. It is interesting that doses of the F.-S.U. extract which by themselves were incapable of increasing the size of the ovary of immature rats, could induce the "augmentation" reaction in the combined injections.

The structure of the ovaries resulting from the combined injections of the F.-S.U. and P.U. extracts in the normal immature rats is different from that resulting from the injection of either of these extracts alone. With P.U. extract injections, as has been often described, the ovaries contain small and medium-sized follicles, hemorrhagic follicles, luteinizing follicles, and corpora lutea. With the F.-S.U. injections large but normal follicles are present. With the combined injections the follicles become luteinized forming the so-called "mulberry ovary," which has the same appearance as ovaries of animals injected with an alkaline extract of the human A.P. (Leonard, 1933).

Discussion. Those familiar with the ovarian effects produced in hypophysectomized rats by injections of A.P. and P.U. extracts and A.P. implants will agree, we believe, that the responses induced by this follicle-stimulating urinary extract are unique.

In this work with hypophysectomized rats we used immature animals so as to avoid the confusing factor of persisting, pre-operatively-formed corpora lutea. We allowed a post-operative interval to elapse before beginning treatments, in order to permit the ovaries to atrophy. This pre-treatment atrophy was pronounced, as shown by the decrease in weights of the individual ovaries from some 0.0075 gram to approximately 0.0030 gram.

The injection of the F.-S.U. extract in these animals induced the maturation of numerous follicles. These were surprisingly uniform in size. In the shorter periods (4–5 days) of treatment, luteinization rarely occurred, and even in the 10-day injections it was slight. It brought the follicles through the stages preparatory to ovulation but did not induce actual rupture. Ovulation occurred in 2 of the 6 animals in which the injections of F.-S.U. extract were followed by an intraperitoneal injection of P.U. extract. The condition to which the F.-S.U. extract brought the follicles seems to be similar to that of rabbits in heat. In them, the follicles are ready to ovulate but an additional stimulus, coitus, P.U. or A.P. injection, is necessary for follicular rupture. The number of follicles, however, matured by the F.-S.U. injections may be greatly in excess of the normal.

The number of eggs ovulated in one of the cases is so greatly in excess of the normal number that it can be characterized as superovulation.

The reparative effect of the injection of F.-S.U. extract strikingly contrasts to the changes which are induced in the ovaries of hypophysec-

tomized animals by the administration of P.U. extract. With P.U. injections, as had recently been described in detail by the authors and by Collip et al., there is no follicular growth. There is, on the other hand, a luteinization of the theca cells and, as we believe, occasionally of the granulosa cells, also. There is also a marked hypertrophy of the interstitial cells which become very similar to lutein cells. These changes which are produced in a 5 to 10 day period of treatment with P.U. extract are the opposite of those induced in the same period by injections of the F.-S.U. extract.

The changes produced in the ovaries of hypophysectomized rats by the injection of F.-S.U. extract differ in several respects from those induced by implants of A.P. With implants, the follicles are very uneven in size, and large cystic ones are almost invariably present (Smith, 1930). They appear to pass rapidly into a cystic condition so that a large number of uniform sized mature follicles are not produced. Even if ovulation could be induced by A.P. implants, the number of eggs liberated would be very small because of the small number of follicles which reach a normal, mature condition at any one time. In the ovaries, with implants, there is also a pronounced luteinization which transforms the follicular cysts into lutein cysts and then into corpora lutea. It is possible that if the treatments with F.-S.U. extract were carried on for a longer period, cyst formation and extensive luteinization would also occur. However, these changes which are very pronounced even with short periods of treatment with implants, are but slight or may be absent with injections of the follicle-stimulating extract for a similar period.

In normal immature rats there are differences between the ovarian changes produced by the P.U. and F.-S.U. extracts at least in the short periods in which we have injected the latter substance. In contrast to the small, medium-sized and hemorrhagic follicles, luteinized follicles, and corpora lutea formed with injections of P.U. extract, the ovaries of normal immature animals injected with F.-S.U. extract contain numerous mature follicles of uniform size.

The ovarian changes induced in the hypophysectomized rats injected with F.-S.U. extract are almost identical with those produced by implants of fresh anterior pituitary in normal immature animals. It will be recalled that Smith and Engle produced superovulation in the latter with short treatments with A.P. implants. The fact that we have also secured superovulation in hypophysectomized rats with injections of F.-S.U. extract, followed by P.U., shows a similar physiological as well as structural condition of the ovaries in the two types of animals.

It seems of much interest that there is a synergistic action "augmentation" between F.-S.U. and P.U. extracts when they are injected concurrently in immature rats as is the case in concurrent injections of P.U.

and an hypophyseal gonadotropic hormone.[6] Although the reaction which results in "augmentation," when P.U. and A.P. extracts are injected concurrently, is controversial, the fact that the F.-S.U. extract can be substituted for A.P. extracts in the production of the phenomenon at least indicates that the two contain a physiologically identical substance. This and the fact that this urine contains a principle which induces reparative changes in the atrophied ovaries of hypophysectomized rats suggests or, as we believe, rather convincingly demonstrates, that the gonadotropic principle in the follicle-stimulating urine is of pituitary origin. It has the physiological characteristics which hypothetically have been postulated for a follicle-stimulating hormone of the anterior pituitary.[7]

SUMMARY

The physiological action of a follicle-stimulating substance occurring in the urine of a woman past the menopause and who suffered from migraine, was studied. Extracts of this urine injected in immature hypophysectomized rats in which a sufficient post-operative period had elapsed for the ovaries to undergo pronounced atrophy, matured a large number of follicles. These follicles were strikingly uniform in size. Even in the longer treatments (10 days) luteinization but rarely occurred. The treatment did not induce an hypertrophy of the interstitial or the theca cells. The reparative effect seemed to be complete. The changes were the opposite in every respect from those induced in hypophysectomized rats by injections of pregnancy-urine extracts.

In two rats in which an extract of the follicle-stimulating urine was injected, a subsequent intraperitoneal injection of P.U. extract induced ovulation. In one case the number of eggs liberated was greatly in excess of the normal number. Oestrin was produced by the ovaries stimulated with F.-S.U. extract.

There was a synergistic action, "augmentation," when an extract of this urine was injected concurrently with a pregnancy-urine extract. In

[6] A similar phenomenon described by Evans et al. (1932) was attributed by them to an activation of the hypophyseal growth hormone. The demonstration of Leonard (1932) that an hypophyseal substance free from growth hormone, but containing a gonadotropic substance, gave "augmentation," compelled the California group to abandon their hypothesis that the growth hormone was "activated." They stated that the pituitary factor responsible for augmentation was free of sex stimulating properties but have claimed in another paper published since this was written that they found an active gonadotropic hormone in their preparation. J. Exp. Med., 1933, vol. 58, p. 545.

[7] The Wisconsin group has long maintained that the anterior pituitary secretes two gonadotropic hormones, a follicle-stimulator and a luteinizer. Their hypothesis has been severely criticized for they have been unable to secure a complete separation of the two in their preparations. Recently, Fevold et al. (1933), however, have added excellent experimental and chemical data in support of their contention.

the production of "augmentation" the extract of follicle-stimulating urine can thus be substituted for the pituitary gonadotropic extract.

The gonadotropic principle in this follicle-stimulating urine appears to fulfill the requirements postulated for an anterior-pituitary, follicle-stimulating hormone.

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ANALYSIS OF CONTRACTION TYPES OF THE EXCISED UTERUS OF THE RAT

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The reaction of smooth muscle to strain was well demonstrated in the early experiments of Straub (1900). In these experiments many other characteristics of muscle were also demonstrated (treppe, summation of stimulation and response). The contractions, however, were smooth and regular. Later (1901) Stewart calls attention to a compound rhythm in bladder muscle. He ascribes this to the independent rhythm of isolated areas, the result being a superimposition of one rhythm upon the other. When a record of this is made by mechanical means the picture presented is that of a compound muscle contraction. This type of response may be observed in almost any series of smooth muscle contractions and may be erroneously thought to be its typical reaction. This cannot, however, be the law of smooth muscle contraction. In the uterine horn of the rat, we find regular contractions at one time and composite (irregular) types at another. The responsibility for this variation we have traced to the intra-uterine tension normally present, or generated during contraction. This paper proposes to identify and analyze typical forms of composite contractions in the excised rat uteri, and show the relation of intra-uterine tension and circular muscle activity to the various forms and phases of a composite contraction.

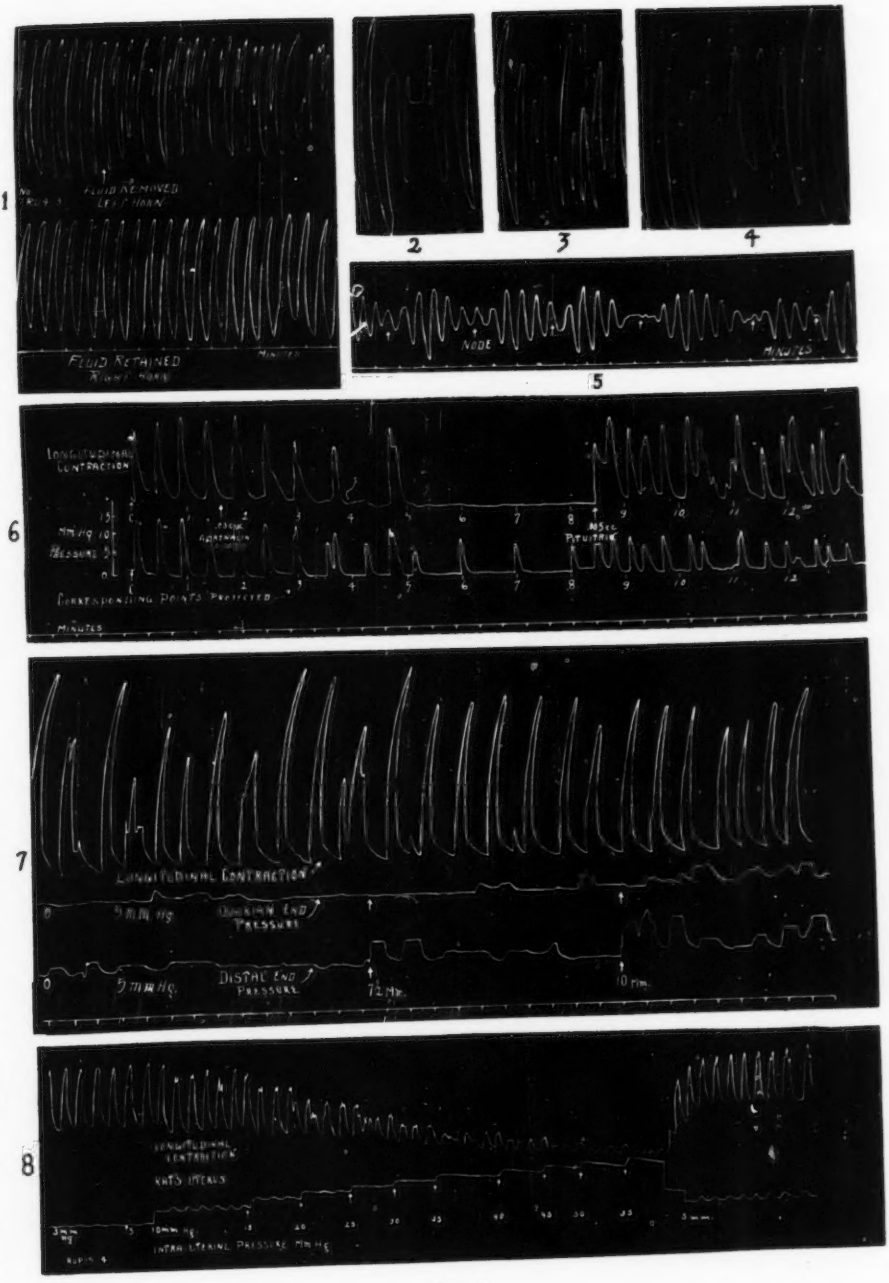
Contraction types in excised rat uteri. In an earlier paper one of us (Harne, 1931) described maximal and submaximal contraction types with respect to the oestrous cycle. In that paper a common form of composite contraction was referred to (p. 232), but was outside the scope of that paper and could not be analyzed by the method used. We (Harne and Painter, 1933) later studied the gradually changing contraction types occurring in uteri of rats from birth throughout life, and following ovariectomy, observing again in the sexually immature, menopause, and ovariectomized animals this irregular type of contraction. The pattern presented in this last type seemed to be very constant under specific conditions (e.g., when the contracting circular muscles temporarily occluded the lumen of the horn, thus forming between contracting rings or in one end of the horn a high local tension), but yielded easily to artificial variations

of intra-uterine tension, e.g., around 15 mm. Hg they become regular, while below 15 mm. they may revert again to the irregular form. In every case where irregular (composite) forms of contraction are normally present the uterine horns have a low intra-uterine tension, and contractions of the circular muscles are apparent. In the following phases of life either one or both of the above conditions exist: 1, stage V of the oestrous cycle (usually low tension only); 2, immature horns, immediately before sexual maturity (around the 45th day); 3, during menopause; 4, during the first two months following ovariectomy.

Since these composite types of contraction are associated with low intra-uterine tension and circular muscle activity, and are only occasionally found in stage V of the oestrous cycle, and never normally in the four preceding stages, we directed our attention first to this variation. It was found that in stage V, if the circular muscles are active, there will be composite contractions of various forms. The distortions always show a direct relation to the circular muscle activity being concurrent with the passage of a peristaltic wave or a maintained high intra-uterine tension. If there are no peristaltic waves, the longitudinal contraction whether maximal or submaximal will be regular. In the four preceding stages of the oestrous cycle the horns are either congested or distended with fluid (uterine milk), and seldom contract in an irregular manner, yet peristaltic waves are clearly discernible. Two possibilities are open, either the circular contractions are weak and ineffective, or the degree of distension of the horn controls the type of response. It was found that in paired horns of this type if the fluid of one be removed, so that the circular muscle contraction can occlude the lumen of the horn and thus produce a high local tension, there will be formed in that horn composite contractions, and simultaneously a new tonus level. The filled horn will be unaffected (1, fig. 1).

Next our attention was directed to the gradually changing types of composite contractions found in the sexually immature, menopause, and

Fig. 1. Showing: 1, the effect of aspirating uterine fluid, upon the contraction type of the excised rat uterus. 2, 3, and 4, various contraction types resulting from the passage of peristaltic waves of various rates and force (see text). 5, the effect when the longitudinal and circular muscle rhythms are not identical but maintained at a fairly constant relation to each other, the result is apparent augmentation and inhibition. 6, the independence of circular muscles to raise intra-uterine tension irrespective of the longitudinal activity. (Note that the rate is almost normal. Numerals indicate projected corresponding points. Note also the timeliness of circular muscle relaxation at the height of longitudinal contraction. Between 8 and 9 it does not occur timely enough and a composite contraction results.) 7, intra-uterine tension at opposite ends of uterus, and the effects of longitudinal contraction upon it under various pressures. Also showing the yielding of the circular muscle to low intra-uterine pressures. 8, the effect of artificially increasing intra-uterine tension upon longitudinal shortening, and the recovery of the tissue.


1
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MINUTES
7
LONGITUDINAL CONTRACTION
PROXIMAL END PRESSURE
DISTAL END PRESSURE
8
LONGITUDINAL CONTRACTION
KATZ'S IDEALS
INTRA-UTERINE PRESSURE MM HG

Fig. 1

ovariectomized animals described by us (Harne and Painter, 1933) in an earlier paper. This form may or may not reproduce itself in a single series, depending upon the effect of circular muscle contractions and its rhythm upon that of the longitudinal. If the two systems maintain a constant relative rhythm, the same pattern will be reproduced again and again as in curves 1 and 5, figure 1, but if the rhythm varies in one and not in the other, the contraction form will change with each contraction, and the influence of the intra-uterine pressure change (either local or general) will be reflected upon the longitudinal contraction in one form or another, e.g., it may be inhibition; an extra contraction may occur, or even an increased rate of longitudinal contraction may result (curves 2, 3, 4 and 5, fig. 1).

The circular muscles produce these effects in a variety of ways: 1. Peristaltic waves may pass rapidly and completely inhibit the longitudinal muscle in any phase of the contraction cycle and also control the events which follow by regulating the intra-uterine tension (e.g., if the intra-uterine tension falls abruptly after inhibiting a longitudinal contraction which is in progress, the longitudinal muscles relax completing a contraction cycle, but if the inhibition passes off, yet maintaining a high intra-uterine tension, the strain excites the muscles and a second contraction occurs no matter whether the cycle is in the contraction or the relaxation phase and an extra contraction is produced). These types of irregular response are illustrated by curves 2, 3, and 4 of figure 1. 2. The contraction wave may start at the ovarian end and slowly pass distally over the horn or only a portion of it. In this type there is no complete inhibition of longitudinal activity as was the case in curve 2, where a succession of waves completely inhibits the longitudinal muscles, but produces a series of steps at points in the contraction cycle where the waves pass (curves 3 and 4, fig. 1). No two successive responses are comparable, but at intervals, very similar forms are observed. 3. Contraction waves may act upon uteri in various states of irritability eliciting the effects (inhibition and excitation) more or less easily, and must be considered as variations in uterine irritability rather than a different type of circular muscle interference. In this type both inhibition and excitation are easily evoked. Curve 4, therefore, is different from curves 2 and 3 only in time relations. 4. Circular muscle rhythm when in phase with longitudinal contraction produces little change in the type of longitudinal response, but when out of phase inhibits or produces distortions of the contraction cycle. In curve 5, an apparent augmentation and inhibition are produced by the two muscle systems beating at constant but different rates—augmentation representing the systems in phase, and the nodes (inhibition) when out of phase. (This curve was produced by treating uteri first with estrogen and later with pituitrin.)

In the five curves thus far discussed we have endeavored to present and analyze typical irregular responses found normally or artificially produced in excised rat uteri of this experimental series without exhibiting proof for all our statements. These curves are, however, most valuable here because they were made with our delicately adjusted apparatus. The responses of the tissues are, therefore, more faithfully reproduced than when our more complicated and less delicate apparatus is used, which we shall discuss presently. Before discussing the remaining five curves where the proof is available and where the intra-uterine pressures are simultaneously recorded with the longitudinal contraction, it will be advantageous to present a brief description of the apparatus used.

Apparatus. The entire apparatus (fig. 2) is constructed from capillary glass tubing designed to hold the uterine horn, *J*, between two cannulae (one for the ovarian and the other for the distal end of the horn) which communicate with small delicately adjusted tambours, *C* and C^1, above which attached levers are made to write upon a kymograph. Immediately above these the lever which records the longitudinal contractions is shown. It is activated by a capillary shaft one end of which serves as cannula, I^1, and communicates with tambour, *C*, through flexible connection, *D*.

The system is of the air transmission type. Locke's solution is provided, however, in bulb *F* for distending the horn and for measuring directly the intra-uterine tension, thus preventing air from coming into contact with the tissue. The tension is measured and the solution manipulated by a standardizing manometer which is attached to connection *H*, figure 2. Any mobility of the Locke's solution in either direction, due to uterine contractions (peristalsis, etc.) will be absorbed by expansion chambers, *E*, thereby controlling hydrostatic shift.

Intra-uterine tension and uterine activity (general consideration). It is clear that the more delicate the recording mechanism the more faithfully it will reproduce those changes which occur. Some delicacy in our recording mechanism was sacrificed when we altered it so as to record simultaneously longitudinal contraction, and intra-uterine tension on both sides of a peristaltic wave, because the finer type curves produced by the longitudinal muscles in curves 2, 3, and 4 are no longer easily obtained. Cruder forms, however, are still present and present graphic evidence similar to that obtained by observation in curves 1 to 5 inclusive. Much additional information is also presented by our latter method and will be cited in a discussion of curves 6, 7, and 8, figure 1, which are continuous records made over 30-minute periods.

Approaching the task of analysis of these curves, one is faced immediately with the fact that in these uteri two muscle systems are potentially active, and may respond independently, or together. The rhythms of activity may be synchronous, or arrhythmical. Adding now to these pos-

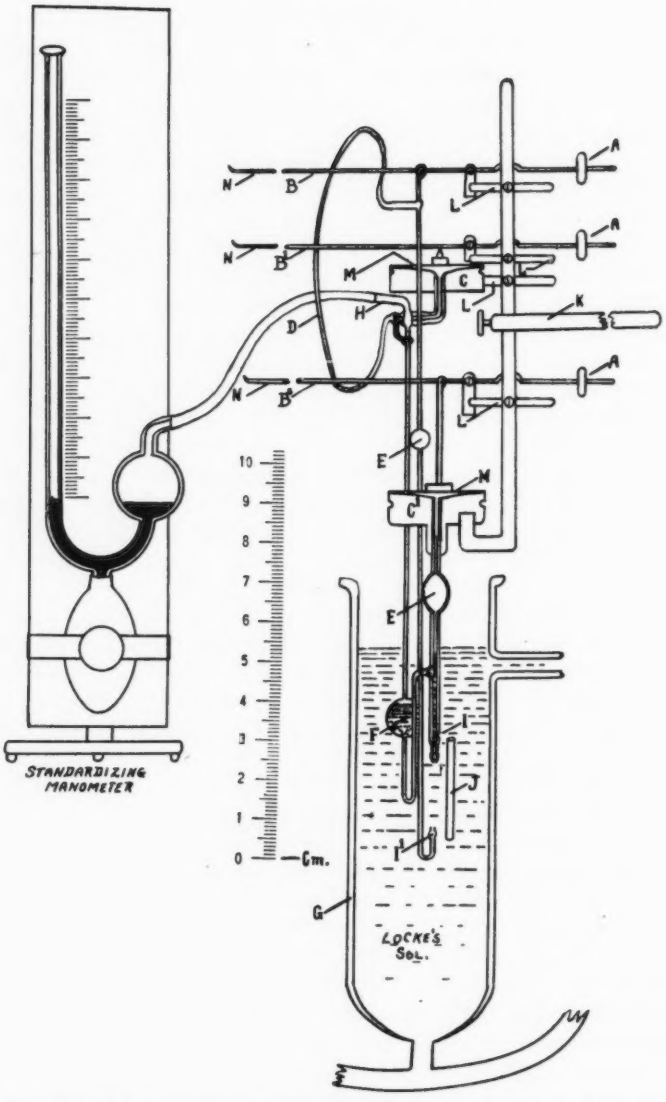

Fig. 2. Showing the apparatus used for the study of intra-uterine pressure and its relation to the phases of a longitudinal contraction. *A*, counter weights on writing levers; *B*, B^1, B^2, writing levers; *C*, C^1, tambours; *D*, flexible connection between cannula I^1 and tambour *C*; *E*, expansion bulbs to absorb shift of Locke's solution in *F*; *G*, muscle chamber; *H*, connection to standardized manometer; *I*, I^1, cannulae; *J*, represents uterine horn; *K*, support; *L*, adjustable levers; *M*, membrane on tambour (rubber); *N*, very flexible writing points.

sibilities the fact that the circular and longitudinal muscles are not of equal strength, and do not survive excision with equality (the circular muscle spontaneity ceasing first), the difficulties ahead may be fully appreciated.

In the few curves here we have endeavored to envelope as much evidence as possible tending to demonstrate these systems at work and show that the pattern of a contraction type is due to the interplay of these mechanisms.

In curves 6 and 7, figure 1, the independent rhythms are demonstrated. The rise in intra-uterine tension (curve 6) during inactivity of the longitudinal muscles shows clearly the independence of the circular muscle. (Note: At this point it should be made clear that under certain conditions, where there is no longitudinal contraction, variations in intra-uterine tension signify circular muscle activity, and also it is conceded that there is circular muscle relaxation if intra-uterine pressure falls during longitudinal activity or rest. This consideration is supported by evidence in curve 7, figure 1. Therefore, under these conditions the terms "intra-uterine tension" and "circular muscle activity" may be used synonymously.) Likewise, also in curve 7 there are longitudinal contractions with and without changes in the intra-uterine tension, thus showing their independence. There are also points where the tension was increased on one side of the peristaltic wave and reduced on the other, showing that the circular muscles occlude the lumen of the horn. This can occur, however, only in the low pressure range (i.e., below 15 mm. Hg intra-uterine pressure) indicating one important characteristic of these circular muscles. Above this pressure the feeble circular muscles contract isometrically, if at all. The ability of the circular muscle to occlude the lumen of the horn, thus dividing it into two chambers has been the criterion by which we have measured circular muscle strength. The yielding of these muscles to relatively low intra-uterine tension may be observed in curve 7, figure 1. The record above 10 mm. Hg is not shown but the tendency of the intra-uterine tension to equalize in both ends of the horn is apparent, and above 15 mm. Hg is complete. From this point upward if the intra-uterine tension be artificially increased, the tension rises and falls in cadence with the contraction and relaxation of the longitudinal muscle. The elasticity of the circular muscles tends, however, to compensate for the grosser changes in pressure brought about by the longitudinal shortening, thereby giving a somewhat linear curve of intra-uterine tension (curve 8, fig. 1).

Intra-uterine tension and the phases of a composite contraction. We may now consider the composite forms of contraction observed in the uteri of the sexually immature, menopause, and ovariectomized rats as being a result of an interplay between the longitudinal contraction and one or more of the phenomena associated with or controlled by the circular muscu-

lature. This interplay may be best understood if we look carefully upon a group of very complex contractions which occurred in a uterus having normally a very low tension, and where at one moment one or more of the foregoing phenomena were active, and at another time were not.

By following the corresponding points on the curve of the longitudinal contraction and intra-uterine pressure curves (9 and 10, fig. 3) we observe that the intra-uterine tension rose (there was a latent period) with the longitudinal contraction, but did not fall with relaxation, thus was maintained a higher intra-uterine tension and the establishment of a new tonus

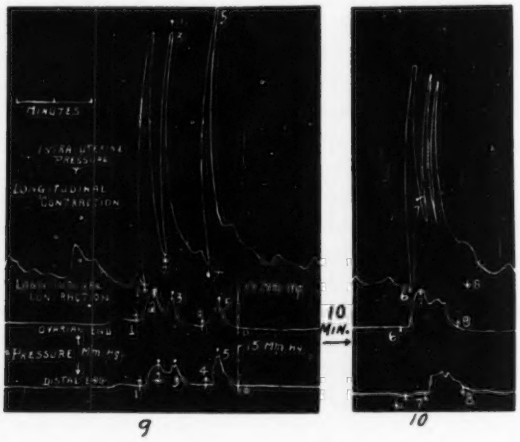

Fig. 3. Showing composite and regular contraction types, and their relation to local and general intra-uterine tension. Numerals represent corresponding points on the various curves.

level. This strain releases again the energy valve of the longitudinal muscle and a second contraction occurs. (Note also in curve 10, fig. 3, that the high tension was local, not involving the entire horn, yet a high tonus level is established and proportionately the contraction rate is increased.) This continues as long as the intra-uterine tension generated is maintained high either generally or locally in the ovarian end, and ends with its release of tension, thereupon the entire system returns to a state of rest. Wherever composite forms occur in this series, they occur coincidently with the passage of a peristaltic wave as heretofore described, or during a period of general or high local intra-uterine tension.

CONCLUSIONS

1. A method and apparatus are described for studying the relationship of intra-uterine tension and contraction types.

2. Irregular or composite contraction types are traced to variations in intra-uterine tension and circular muscle activity.

3. The pattern of the composite contraction type depends upon the position in the contraction cycle where the phenomena produced by the circular muscle activity have their effect.

4. Varieties of these composite contraction types were observed and analyzed.

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THE EFFECT OF DIVERTING THE ADRENAL VEIN BLOOD INTO THE PORTAL VEIN ON THE BLOOD SUGAR OF DOGS[1]

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The intravenous injection of epinephrine has been demonstrated to cause a temporary hyperglycemia. It seems probable that this effect is chiefly due to hepatic glycogenolysis. The present study represents an attempt to determine the effect on the blood sugar level of producing a permanent diversion of the blood from the adrenal glands into the portal vein. Epinephrine is fairly rapidly destroyed or inactivated in the circulating blood and it might be supposed that an alteration in the circulation so that the adrenal veins were made to empty into the portal system instead of the vena cava would produce some definite changes in carbohydrate metabolism.

The experiment was performed in the following manner. Attempts to implant the adrenal veins directly into the portal vein were unsuccessful because of technical difficulties. A successful result was secured by the series of operations indicated in the diagram in figure 1. All of the operations were performed on dogs under complete ether anesthesia and with strict aseptic precautions. At the first operation a lateral anastomosis was made between the vena cava and the portal vein and the cava was tied off with heavy linen thread immediately above the anastomosis. This procedure was found to cause a definite elevation of the blood sugar beyond that produced by other surgical operations under the same anesthesia. The data are graphically illustrated in figure 2. One cannot be certain however that the adrenal vein blood in these dogs would continue to pass to the liver since the development of collateral venous circulation might reverse the current in the vena cava. To control this possibility at a second operation performed several weeks later the vena cava was tied off distal to the entrance of the renal veins. In order to prevent a reversal of flow in the lumbo-adrenal veins these were tied off distal to the adrenal glands. The effect of these procedures on the level of the blood sugar is illustrated by the curves of two representative experiments in figure 3. In each case a definite hyperglycemia was produced as well as marked

[1] A preliminary report of this work was made at the 43rd meeting of the American Physiological Society in Montreal, April 1931.

fluctuations in the blood sugar level from day to day. The average blood sugar before and after operation may be summarized as follows: dog 1, before operation 78, after operation 128; dog 2, before operation 81, after operation 100; dog 3, before operation 79, after operation 88; dog 4, before operation 80, after operation 107. The animals recovered promptly from the effects of the operation and have continued in good health until the present time or about five years. A second examination was made 18 months after the original operation. The average blood sugar level of dog 2 for 30 days was 85 with a daily variation of ± 5 per cent, of dog 3 was 83 with a daily variation of ± 7 per cent, and of dog 4 was 99 with a daily variation of ± 11 per cent. Thirty-two daily determinations of the blood sugar were made on each of three normal dogs kept in the same

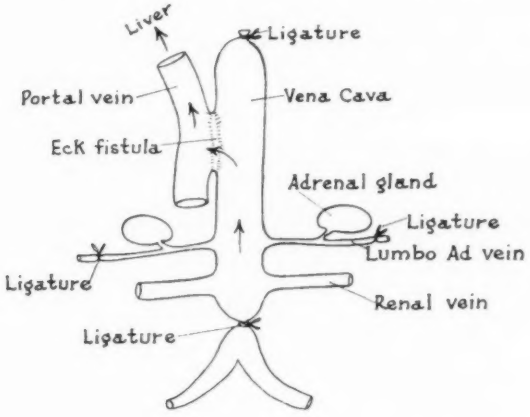

Fig. 1

laboratory under similar conditions and yielded an average of 84, 87, and 75 with a daily variation of ± 4 per cent. Five years after the original operation the blood sugar of the surviving animals was again determined with the following result: dog 2, 75 mgm. (average of 5 determinations), and dog 4, 106 mgm. (average of 5 determinations). All of the sugar determinations were made by the method of Folin and Wu (1920) on blood samples secured from 18 to 24 hours after the last feeding.

The data clearly indicate that the hyperglycemia produced in animals 1, 2, and 3 was of only temporary duration and the blood sugar returned to its normal level within 18 months. Animal 4 however continued to display a fasting blood sugar with an average of 20 mgm. above the preoperative level until it was sacrificed 5 years after the original operation. The autopsy findings add considerable significance to these results. In

the animals whose blood sugar returned to normal (1, 2, and 3) it was found that the ligature about the vena cava above the Eck fistula had dis-

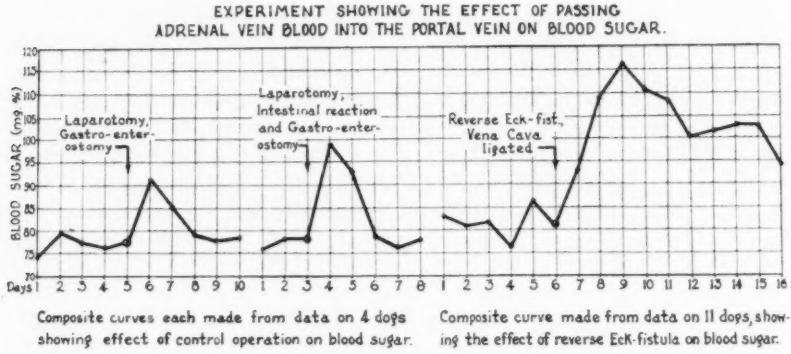
EXPERIMENT SHOWING THE EFFECT OF PASSING
ADRENAL VEIN BLOOD INTO THE PORTAL VEIN ON BLOOD SUGAR.
BLOOD SUGAR (mg %)
Laparotomy, Gastro-enterostomy
Laparotomy; Intestinal reaction and Gastro-enterostomy
Reverse Eck-fist., Vena Cava ligated
Days
Composite curves each made from data on 4 dogs showing effect of control operation on blood sugar.
Composite curve made from data on 11 dogs, showing the effect of reverse Eck-fistula on blood sugar.

Fig. 2

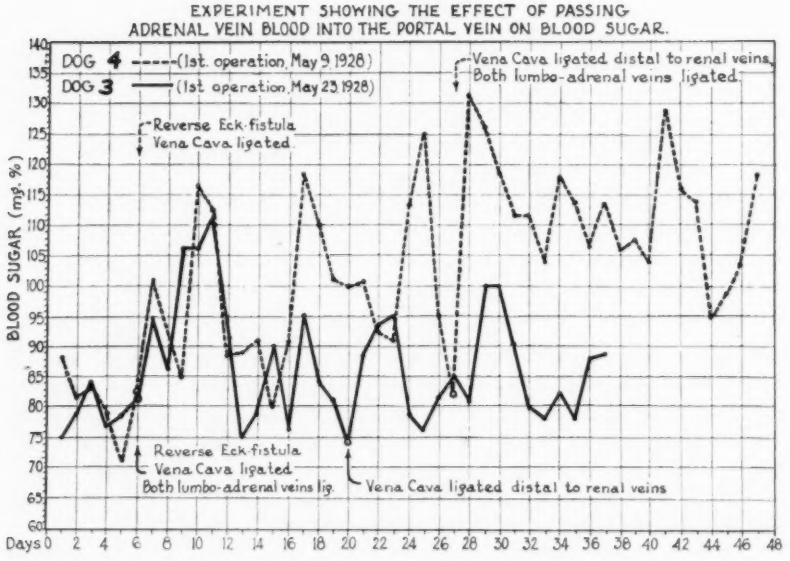
EXPERIMENT SHOWING THE EFFECT OF PASSING
ADRENAL VEIN BLOOD INTO THE PORTAL VEIN ON BLOOD SUGAR.
DOG 4 -----(1st. operation, May 9, 1928)
DOG 3 ——(1st. operation, May 23, 1928)
Vena Cava ligated distal to renal veins, Both lumbo-adrenal veins ligated.
Reverse Eck-fistula Vena Cava ligated.
Reverse Eck-fistula Vena Cava ligated Both lumbo-adrenal veins lig.
Vena Cava ligated distal to renal veins
BLOOD SUGAR (mg. %)
Days

Fig. 3

appeared and the lumen of the vena cava had become reëstablished. The anastomosis with the portal vein was still patent but greatly reduced in size. In dog 4 however the vena cava was completely obliterated above

the Eck fistula and below the renal veins so that all blood from the kidneys and adrenal glands had to pass directly to the liver.

The tolerance of these various animals to the intramuscular injection of insulin was determined shortly before they were sacrificed. No significant alterations were found. Dog 4 displayed no greater resistance to hypoglycemia or convulsions than the normal animal.

All animals were sacrificed by electrocution 24 hours after the last feeding. Samples of the livers were immediately removed for the determination of liver glycogen by the method of Cori (1932). Eleven and five-tenths milligrams of glycogen per gram of wet liver were found in dog 4 as against 20.5 mgm. and 25.0 mgm. in two controls.

SUMMARY

A method for producing a permanent diversion of adrenal vein blood into the portal vein in dogs has been described. Such a diversion was found to cause a moderate persistent elevation and instability in the level of the blood sugar. Glycosuria was not produced and no increased tolerance to insulin was observed.

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THE ACTION OF CALCIUM AND POTASSIUM IONS ON THE NICTITATING MEMBRANE, THE ADRENAL MEDULLA AND THE NON-PREGNANT UTERUS OF THE CAT

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The abundant literature existing on the effects of Ca and K ions (see Zondek, 1927, for references) deals principally with studies on excised structures. It was deemed desirable to examine the action of these ions on organs normally supplied with blood. The smooth muscles chosen were the nictitating membrane (n.m.) and the non-pregnant uterus (n-p.u.) of the cat, both of which are exclusively supplied by sympathetic nerve fibers which evoke pure excitation in the n.m. and pure inhibition in the n-p.u. The results obtained led us to extend the study to the effects of the ions on medulliadrenal secretion.

METHOD. Dial anesthesia or decapitation under ether was employed. The latter was performed through the foramen magnum after ligation of the left carotid and temporary (about 2 min.) closure of the right carotid with a bulldog clamp and compression of the vertebrals by hand. When the clamp on the right carotid was released a normal circulation to the right n.m. was reëstablished.

The contractions of the n.m. were recorded isotonically. When the two membranes were used in animals under dial, the records were obtained as described by Rosenblueth (1932). The responses of the denervated n-p.u. were recorded by the method used by Cannon and Rosenblueth (1933).

The solutions of $CaCl_2$ and KCl (5 per cent) and of adrenalin (0.001 per cent) were injected into the femoral vein. Curare was used in the pithed preparations to abolish perturbing movements of spinal origin. Cocaine was employed to increase the responses to injected adrenalin and secreted adrenin. Artificial respiration was invariably given to eliminate the influence of asphyxia.

RESULTS. When the adrenals are present and innervated typical sympathomimetic effects ensue on injections of either $CaCl_2$ or KCl—i.e., there is a contraction of the n.m. and an inhibition of the n-p.u. (fig. 1A and B). This inhibition is sometimes followed by a slight contraction, the origin of which will be explained below. These responses, however, are not due

[1] Advanced Fellow of the C. R. B. Educational Foundation.

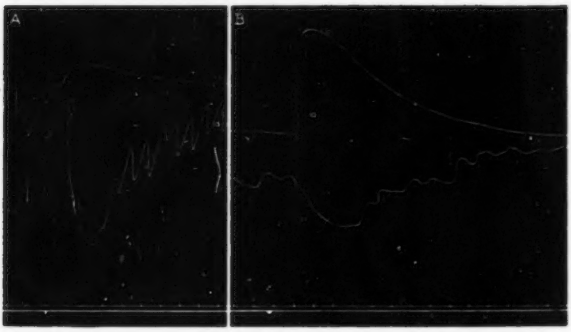

Fig. 1. Brain pithed. Curare. Cocaine. Adrenals intact. Upper record: acutely denervated nictitating membrane; lower record: denervated non-pregnant uterus. In this and other figures the time is recorded in 30-second intervals.

A. Effects of injecting 0.3 cc. of 5 per cent $CaCl_2$.

B. Effects of injecting 0.5 cc. of 5 per cent KCl.

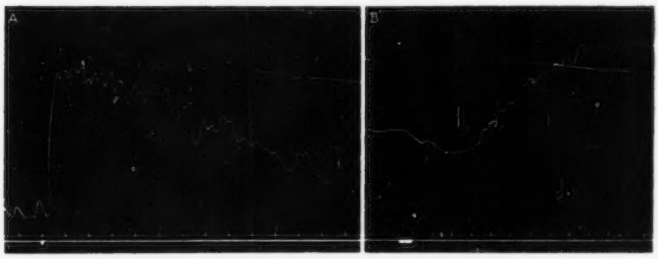

Fig. 2A. As in figure 1, but adrenals ligated. Effects of injecting 1 cc. of 5 per cent $CaCl_2$.

B. As in figure 1, but right adrenal ligated and left adrenal denervated. Effects of injecting 1 cc. of 5 per cent KCl.

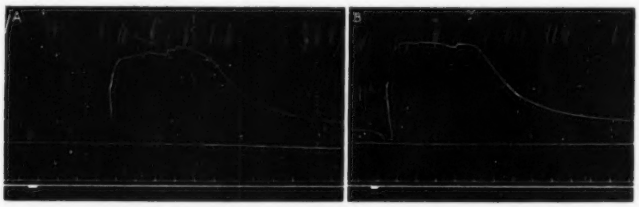

Fig. 3. Right nictitating membrane (upper record) denervated postganglionically 7 days before the experiment. Left nictitating membrane (lower record) denervated acutely. Dial. Adrenals ligated.

A. Effects of injecting 1 cc. of 5 per cent $CaCl_2$.

B. Effects of injecting 0.5 cc. of 5 per cent KCl.

to a direct effect on the smooth muscles, but to a secretion of adrenin elicited by the ions. If the adrenals are ligated previously, Ca evokes a contraction of the n-p.u. and no response from the acutely denervated n.m. (fig. 2A), and K produces a contraction of both the n-p.u. and the n.m.

The secretion of adrenin is due to both central and peripheral excitation by the ions. This was determined by removing the right adrenal and the left abdominal sympathetic chain above the kidney. This procedure leaves the left adrenal still innervated by the splanchnics. Both Ca and K gave the effects described above (fig. 1). The left splanchnics were then cut; the effects of the ions on the n.m. persisted but were markedly decreased, while the n-p.u. still responded by lessened inhibition followed by contraction (fig. 2B). This inhibition of the n-p.u. could only be due to adrenin secreted by the denervated gland, for it disappeared entirely on removal of the left adrenal.

The contractions of the acutely denervated n.m. on administration of K ions require large doses (2 cc. or more of the 5 per cent solution), whether cocaine is present or not. If one of the membranes is denervated previously by removal of the superior cervical sympathetic ganglion and a sufficient period is allowed for degeneration of the postganglionic fibers (usually about 6 days), both Ca and K will readily evoke a contraction, whereas the acutely denervated n.m. responds minimally or does not contract at all (fig. 3A and B). These effects are obtained with the adrenals ligated. We conclude, therefore, that both Ca and K elicit a contraction of the n.m. and that the degeneration of the postganglionic sympathetic fibers greatly augments the responses, as it augments the responses to adrenin and to other substances (Rosenblueth, *loc. cit.*).

The effects of the ions on adrenal secretion are transient (usually from 1 to 3 min.). The responses of the n.m. and the n-p.u. to both Ca and K are longer; the contraction of the n-p.u. after Ca may last 30 minutes or more, especially if cocaine has been previously injected. Successive doses of the same ion produce increasing effects. This makes it difficult to determine whether cocaine increases the responses. One gathers from the experiments, however, the impression that such is the case.

When either the n.m. or the n-p.u. is contracted as a response to Ca or K, it is not refractory to adrenalin. Indeed, after Ca the responses to adrenalin are usually increased (cf. Trendelenburg, 1916). Injections of Na citrate or oxalate, which inactivate or precipitate Ca ions, inhibit temporarily the contraction obtained from Ca. When either Ca or K and adrenin act simultaneously, an algebraic summation of the responses occurs, as evidenced by comparison with the effects of injected adrenalin.

Discussion. The results obtained on the n-p.u. confirm those reported by Tate and Clark (1922) on isolated uteri.

The responses recorded are due to the effects of the ions on the centers and structures studied and not secondary to circulatory changes. The heart beat was invariably examined and found satisfactory after the injections. Changes of blood pressure produced by compressing and releasing the thorax evoke practically no change in length of either the n.m. or the n-p.u.

The central effects of the ions on autonomic innervation appear to be restricted to the splanchnics with the doses used. There never was any outward sign of sympathetic activity elsewhere (e.g., pilomotors). A possible central effect on the sympathetic supply to the n.m. was investigated by recording the responses of the two n.m's., one acutely denervated and he other innervated. No noticeable differences occurred, even after cocaine, which sensitizes the responses to sympathetic nerve impulses (Rosenblueth and Rioch, 1933).

The results presented obviously do not permit a classification of either Ca or K ions as sympatho- or parasympathomimetic. Numerous exceptions to Zondek's law have been reported (see Bacq, 1932, for references). In the three organs studied there is no antagonism between Ca and K. This might be correlated with the fact that all three are exclusively under control of the sympathetic system.

SUMMARY

The effects of intravenous injections of $CaCl_2$ and KCl on the nictitating membrane, the non-pregnant uterus and medulliadrenal secretion were studied in cats.

Both ions have a stimulating action on medulliadrenal secretion (fig. 1). This action is both central, through the splanchnics, and peripheral, on the chromaffine cells of the gland.

Both ions have a sympathomimetic action on the nictitating membrane, evoking a contraction. This effect is peripheral and is increased by degeneration of the nerve supply to the muscle (fig. 2).

Neither ion has an autonomomimetic action on the non-pregnant uterus, since they both elicit a contraction (fig. 2) and there are no nerves known to have a similar effect.

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THE JAW-JERK AND ITS AFFERENT PATH

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Jaw-opening and jaw-closing reflexes, obtained by various methods of stimulation, have been described for the decerebrate cat by Sherrington (1906) and Bremer (1923). The true "jaw-jerk," or stretch reflex of the jaw-closing muscles, is typical of the "jerk phenomena" of the decerebrate state. In the present investigation, myographic and electromyographic studies have been made of the jaw-jerk, as well as a functional study of its afferent path.

METHODS. Cats decerebrated by the trephine method were used. Transection of the brain stem was made through the middle of the anterior colliculi two to three hours before the records were taken. The masseter muscle on the right side was then carefully exposed for the insertion of electrodes. The myographic set-up and galvanometer used were the same as those described by Forbes, Davis and Lambert (1930), except that the isometric lever was considerably lighter in construction. The natural period of the myograph was 425 cycles per second. One kilogram of tension gave 50 mm. excursion on the film.

With the cat in the dorsal position the skull was clamped securely to the supporting rod, in such a position that a strong thread could be attached to the lower canine tooth, passed around a pulley below and fastened to the lever directly above. The desired degree of initial tension could be thus obtained and the movements of the jaw recorded.

The jerk was elicited by a sharp tap upon the first molar tooth with a moderate initial tension of the jaw-closing muscles. Action currents were recorded with chlorided silver-needle electrodes inserted into the masseter muscle.

Inhibition of the jerk was obtained by stimulating the buccal mucosa with a single break shock from a Harvard inductorium. Maximal stimuli were used. Successive jaw-jerks at intervals of 0.8 second were elicited and the inhibitory stimulus applied at random.

In the acute experiments for studies on the reflex arc, the cats were decerebrated in the same manner as described above. The jaw was sawed through at the symphysis and the soft parts divided in the mid-line to the

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level of the angle of the mandible. The jaw-jerk was elicited either by a brisk tap on the canine tooth or on the skull, with the jaw-closing muscles held moderately stretched. The degree of tonic contraction present in these muscles was roughly noted. The fifth nerve was then exposed on one side, at its point of emergence from the brain stem, by removing part of the bony tentorium. The sensory root was cut approximately mid-way between its point of exit and the Gasserian ganglion. The motor root was left intact. The jerk was tested again and the muscle "tone" noted.

In three cats an attempt was made to sever the sensory root, under aseptic conditions for surviving preparations. It was necessary to remove the occipital pole of the cortex in order to permit removal of part of the bony tentorium. An examination similar to that used in the acute experiments was made three weeks later.

RESULTS. *Myographic studies.* Figure 1A shows a typical record of the jaw-jerk and its action current. The down-stroke, *m*, in the myograph record indicates the mechanical stimulus. It is followed by a response, *r*, similar to that of a typical motor twitch except that the relaxation phase is longer. This represents the increased tension of the jaw-closing muscles. The contraction time of the motor twitch of the masseter muscle, measured from the beginning of contraction as indicated by the lever, to the height of contraction, is 15 to 20 σ, whereas that of the reflex measured in the same way is 25 to 28 σ. If measured from the beginning of the action current, the reflex contraction time is 32 σ. No action currents accompanying the twitch were recorded.

The action current, *a.c.*, is recorded with a moderately slack string. Its latent period is from 7 to 10 σ, with an average of 8.5 σ. There are at least two closely spaced volleys of impulses seen in the normal, uninhibited action currents, indicated by the double peaks. Action currents following inhibitory stimuli (fig. 1C and D) show, in certain cases, only a single response.

The effects upon the jaw-jerk of inhibitory stimuli, applied at various intervals preceding the mechanical stimulus, are shown in figure 1B, C and D. At intervals above about 120 σ the inhibitory stimulus is barely effective. Complete inhibition is obtained with intervals less than 16 to 18 σ. No effect upon the following jerk is apparent, approximately 0.8 second later.

Studies on the afferent path. In four acute experiments, severing the sensory root abolished the jaw-jerk and there was complete flaccidity of the jaw-closing muscles, on the side of the lesion. The motor root on the same side was stimulated at a point central to the section of the sensory root, and a normal contraction of the masseter muscle was observed. To rule out spread of current, the motor root was then cut and the same stimulus was applied to the same region. No muscular response was obtained.

In the chronic experiments only one successful operation was performed. In this case the sensory root was completely severed, as shown by anesthesia to pin-prick over the left side of the face, and complete absence of the left corneal reflex. After decerebration performed three weeks later, the left jaw-jerk was absent, the right active. There was flaccidity of the left

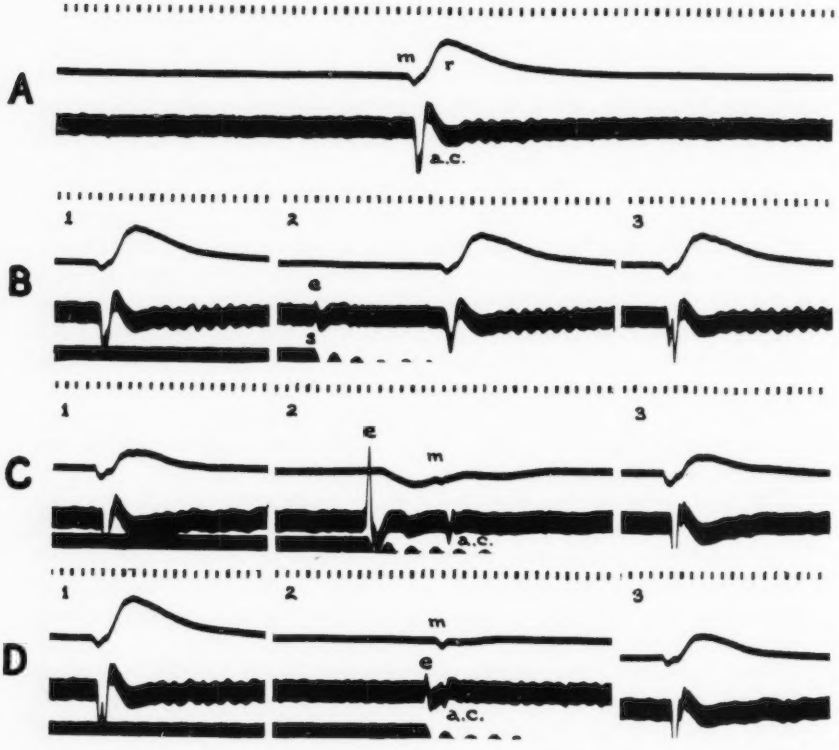

Fig. 1A. Myographic and galvanometric record of jaw-jerk. *m* = mechanical stimulus; *r* = muscular response; *a.c.* = action current. Time recorded at intervals of 10 σ.

B, C and D. Records of successive jaw-jerks (1, 2 and 3) at approximately equal intervals which average 860 σ. The middle records (2) show the effects of an inhibitory stimulus applied at various intervals. *e* = electrical artefact of inhibitory stimulus; *s* = signal for inhibitory stimulus.

B. Virtual absence of inhibition Interval between stimuli 115 σ.

C. Partial inhibition. Marked decrease in *a.c.* Interval 65 σ.

D. Inhibition almost complete. Interval 18 σ.

jaw-closing muscles, though atrophy was not evident. Stimulation showed the motor root on the left to be intact. When this was cut, there was no increase in flaccidity. Marchi sections of the mandibular branch of the fifth nerve in this case showed practically no degenerated fibers. There were a few scattered degenerated fibers in the sections made of the Gasserian ganglion.

DISCUSSION. The data indicate that the jaw-closing muscles are *fast* muscles (Denny-Brown, 1929). The contraction time of the masseter and temporal is probably the same, since the myogram is smooth. They are faster than the muscles of the limbs, the fastest of which have contraction times of 40 σ as recorded by Cooper and Eccles (1930).

The jaw-jerk is not a "biting" or "chewing" reflex, but rather a response to stretch. It therefore differs from those responses, described by Sherrington (1917), to stimulation of the mucosa or pressure upon the molar teeth. It differs in this respect also from that reflex described by Bremer, which is evoked by pulling down the corners of the mouth.

The resemblance between the decerebrate knee jerk (Fulton, 1926) and the jaw-jerk is quite striking. The jaw-jerk is elicited by stretch, it has a short latent period and it is inhibited with difficulty. It is therefore typical of other myotatic reflexes in antigravity muscles in the decerebrate state. This furnishes quantitative evidence for Sherrington's statements regarding the "jerk phenomena " (Sherrington, *loc. cit.*).

On anatomical grounds, several authors (Willems, 1911; Allen, 1919; and others) have postulated that the mesencephalic root of the fifth nerve is the proprioceptive center for the muscles of mastication. This has been questioned by Bremer (*loc. cit.*), who showed that the jaw-jerk is not abolished by section of the mesencephalic root. The results of Bremer were confirmed by Matthes and D. McK. Rioch (1930, unpublished).[2] It is generally conceded that the mesencephalic root fibers leave the brain stem mixed with the motor root fibers (for literature see Weinberg, 1928). The fact that Marchi sections in the survival preparation showed no degeneration in the peripheral nerve is sufficient evidence that in this case the descending mesencephalic root fibers were intact. If so, and if the mesencephalic root is proprioceptive in function, cutting the sensory root should not interfere with the myotatic reflex. However, as stated above, the jaw-jerk and the tonic contraction of the muscle are abolished by cutting the sensory root at the time of decerebration or three weeks previously. It is thus evident that the mesencephalic root is not essential and that the sensory root is essential for the myotatic reflex. This makes it extremely improbable that the neurones of the mesencephalic root are concerned with this proprioceptive function.

[2] Unpublished communication by D. McK. Rioch.

SUMMARY AND CONCLUSIONS

1. Myographic and action-potential studies of the jaw-jerk are presented. The contraction time of the masseter muscle, 15 to 20 σ, classes it as a *fast* muscle. The reflex resembles a motor twitch. The latent period of the action current is 8.5 σ.

2. Complete inhibition of the jaw-jerk is only obtained if the inhibitory stimulus precedes it by less than 16 to 18 σ. Partial inhibition is effective up to 115 to 120 σ.

3. The afferent path for the jaw-jerk in the decerebrate cat is through the Gasserian ganglion and the sensory root.

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THE EFFECT OF EXERCISE ON THE ACETONE BODIES IN THE BLOOD OF MAN ON LOW CARBOHYDRATE DIET

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Evidence for or against the utilization of fat during muscular exercise in man has been obtained from observations of the respiratory quotient, the blood fats and the acetone bodies. The respiratory quotient has been most extensively studied. Rapport (1930) has reviewed the literature on the respiratory quotient of the exercising animal. He came to the conclusion that the evidence from the respiratory quotient did not justify the belief that carbohydrate alone was oxidized to supply the energy for muscular activity. Since Rapport has summarized the work in this field it is not necessary to review it again for this paper. Recently, doubts have been raised as to the respiratory quotient being a true indicator for combustion of particular foodstuffs. Cathcart and Markowitz (1927) claim that the non-protein respiratory quotient is not a true combustion quotient but is the algebraic sum of the transformation of carbohydrate to fat and fat to carbohydrate plus the oxidation of carbohydrate for energy purposes. Thunberg (1930) considers that none of the oxygen consumed in general metabolism is found in the expired carbon dioxide. The oxygen used during respiration is transformed to water. Therefore the relationship of carbon dioxide to oxygen in the expired air has no bearing on the actual metabolic processes in the body. Since there are two ways of interpreting the respiratory quotient, the evidence obtained from this quotient in man during exercise becomes less valid for determining a particular type of metabolism.

Changes in the blood fats during and after exercise have not been extensively studied. Patterson (1927), using the method of Stewart and White (1925), found an increase in blood fats during work. Since Long and Venning (1932) have shown that this method also determines organic acids formed from glucose, these experiments should be repeated with a better method.

Numerous attempts have been made to correlate the changes in the acetone bodies in the urine with exercise in man. Hirschfeld (1895) found no marked change in the acetone content of the urine following exercise in

two subjects on carbohydrate-low diets. von Noorden (1907) studied the effect of exercise in two diabetic patients. He found a slight rise in the acetone of the urine in one subject following exercise and a fall in the second subject. Forssner (1909) and Preti (1911) found marked increases in the output of acetone in the urine of men on low carbohydrate diets, the rise continuing for some time after the exercise was over. McClellan and Toscani (1928), while studying the rate of excretion of acetone bodies during the day in men on low carbohydrate diets found a higher rate in the afternoon than in the morning. They attributed this rise to their subjects exercising in the afternoon. There was a lag in the rate of excretion of the acetone in the urine, the maximum rise occurring a few hours after the cessation of exercise.

With the growing tendency to discredit the respiratory quotient in man as a true indicator of particular metabolism, the lack of good evidence from blood fat determinations, and the discrepancy between the results of the effect of exercise on the acetone bodies in the urine and the complicating factor of kidney excretion in these experiments, it was thought that a better analysis of the problem of fat utilization during work would be obtained by making a study of the acetone bodies in the blood of man before and after exercise on normal and low carbohydrate diets.

Methods. The determinations of the total acetone bodies in whole blood using 5 cc. samples were made with Hubbard's (1921a) method, using the recent modifications given by Peters and Van Slyke (1932). All the precautions suggested by Hubbard were observed. The solutions were shaken in a shaking machine for ten minutes after the addition of each reagent for the removal of protein and sugar. The flasks used for the final distillation were boiled with a solution of sodium peroxide before using. Condensers and flasks with interchangeable glass connections were used in all of the determinations so that the solutions did not come in contact with cork or rubber stoppers. With each experiment a blank determination was made on the entire method and another on the final titration. One additional step was introduced into the method. Difficulty was experienced in obtaining a clear solution after centrifuging the mixture of blood, water, colloidal iron, Goulard's reagent and sodium hydroxide as all of the colloidal iron did not precipitate. This was overcome by the addition of ten cubic centimeters of 20 per cent $CuSO_4 \cdot 5H_2O$ after the addition of Goulard's reagent, shaking for ten minutes and adding sufficient 2N NaOH to remove the iron, lead and copper salts. This amount of copper sulfate is the same as Hubbard (1921b) used in his determination of acetone bodies in the urine. Preliminary analyses were made on blood to which 0.2 mgm. of acetone was added, 95 per cent of the added acetone being recovered. Duplicate analyses were made on blood samples drawn from a subject on a normal diet and also after four days of low carbohydrate diet. Three such

determinations on normal bloods showed variations of 0.02, 0.03 and 0.03 mgm. per 100 cc. of blood between the duplicate analyses. Using bloods from a subject on a low carbohydrate diet, variations of 0.17, 0.20 and 0.04 mgm. per 100 cc. of blood were found between samples. Therefore, changes greater than 0.20 mgm. per 100 cc. are significant. Potassium citrate was used as an anticoagulant.

The experimental procedure on each subject was as follows: One or two experiments were done with the subject on his usual diet to see if any marked changes occurred normally in the acetone bodies of the blood during or following exercise and also to accustom the subject to the experimental routine. For these determinations, the subject came to the laboratory without breakfast and rested on a bed beside the bicycle for one hour. At the end of this time a sample of blood was taken from an arm vein. The subject then pedalled a stationary bicycle for one hour at a constant rate and against a constant frictional force. On returning to the bed, another blood sample was obtained. In subjects R. L. and J. C. a third sample was taken one hour after the work had been completed. Riding the bicycle at this rate and load probably increases the metabolism four to five times as Gemmill, Booth and Pocock (1930) found metabolic rates around 1200 cc. of oxygen per minute for slightly greater work on this bicycle.

Following this experiment, the subject commenced the low carbohydrate diet. It consisted mainly of bacon and eggs, 40 per cent cream in coffee, butter, pork and lamb chops. On the fourth day of the diet, a similar work experiment was carried out with the exception that a longer recovery period was obtained. In C. G. a two-hour period, in R. L. a three-hour and in J. C. a four-hour recovery was studied.

Results. The results of all the experiments are given in graphic form in figures 1 and 2 and a detailed summary of the experiments on C. G. in table 1. In table 2 the amounts of work done by each subject are given in kilogram-meters per minute.

There was practically no change in the acetone bodies of the blood following exercise in the three subjects on a normal diet. In contrast, when the subjects were on a low carbohydrate diet, a definite increase of the acetone bodies was found after exercise. The maximal rise came two hours after the exercise was over. It is interesting to note the differences in the resting level of acetone bodies in the three subjects on a low carbohydrate diet for the same period of time (fig. 2).

In order to see if there was any marked variation in the acetone bodies in a resting subject on a low carbohydrate diet over the same period of time taken for the exercise experiments, subject C. G. again went on this diet. On the fourth day of the diet blood samples were drawn from the subject at the end of the first, fourth and six hours of a basal period. The

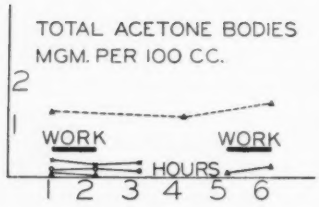

Fig. 1

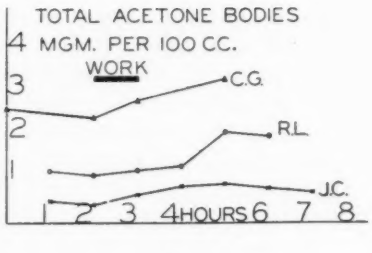

Fig. 2

Fig. 1. Solid lines: Effect of exercise on acetone bodies in blood from subjects on normal diets.

Dotted line: Variation in acetone bodies in blood from a basal subject on a low carbohydrate diet.

Fig. 2. Effect of exercise on acetone bodies in blood from three subjects on low carbohydrate diets.

TABLE 1

Summary of all determinations made on C. G.

EXPERIMENT	DATE	DIET	TOTAL ACETONE BODIES MGM. PER 100 CC. WHOLE BLOOD					
			Hours					
			1	2	3	4	5	6
1	October 21	Normal	0.10	0.25				
2	October 22	Normal	0.11	0.05				
3	October 26	Low carbohydrate	2.64		2.45	2.88		3.37
4	November 17	Low carbohydrate	1.56			1.40		1.72

In experiments 1 and 2 the work was done between the first and second hours; in experiment 3 between the third and fourth hours. No work was done in experiment 4.

TABLE 2

Rate of working in kilogram-meters per minute

EXPERIMENT	SUBJECT	DIET	WORK KGM.-M. PER MINUTE
1	C. G.	Normal	334
2		Normal	332
3		Low carbohydrate	312
4	R. L.	Normal	311
5		Low carbohydrate	304
6	J. C.	Normal	332
7		Low carbohydrate	332

The individual data for the three subjects is as follows: J. C., age 35 years, height 183 cm., weight 75 kgm.; C. G., age 32, height 180 cm., weight 82 kgm.; R. L., age 30, height 173 cm., weight 63 kgm.

results are given by the dotted line in figure 1. The slight rise observed over this period of time is not comparable to the increase found after exercise.

Subjects C. G. and R. L. complained of marked symptoms of fatigue while pedalling after the low carbohydrate diets. Other observers (Krogh and Lindhard, 1920; Reynolds et al., 1927) have also recorded that their subjects were more readily fatigued on low carbohydrate diets.

Discussion. It is commonly accepted that the acetone bodies in the blood of men on low carbohydrate diets, in starvation and in diabetes come from the incomplete combustion of fat. Therefore, an increase in these bodies resulting from work suggests that there has been an increase in the amount of fat broken down during and after muscular work to supply the energy for the exercise. The exact mechanism for the utilization of this fat cannot be obtained from this type of experimentation, except that it does give suggestive evidence that the fat is oxidized directly and not converted into carbohydrate. Also the explanation of the late rise in the acetone bodies must await future experimentation. It may mean that the fats are used in the recovery period of exercise, possibly to supply the energy for the reconversion of the lactic acid into glycogen. Although the exercise was of such nature as not to produce a marked oxygen debt, the duration of the recovery period may be different on low carbohydrate diets from that on normal diets.

Several objections may be raised to the suggestion that these experiments give evidence that fats are used directly for energy production during muscular exercise. The fuel for muscular exercise under these conditions may have come from residual carbohydrate stores. With the increased depletion of the carbohydrate supply, the level of acetone bodies might be gradually raised. The curves for R. L. and especially for J. C. show a maximum at two hours after exercise and then a partial return to the base line. If the general level of acetone bodies was being raised through additional depletion of carbohydrate stores, there should be a continuous rise and not a late decrease. There is also the possibility that the increase in acetone bodies may come from the breakdown of proteins for it has been shown that some amino acids may give rise to aceto-acetic acid and β-hydroxybutyric acid. This work has been summarized by Dakin (1912). It would be extremely difficult to prove or disprove in man that this rise of acetone bodies resulted from protein metabolism. Therefore the more general view is accepted that the acetone bodies represent incomplete products of combustion of fats.

It may even be argued that the combustion of fats may result from a stimulus arising from some chemical change taking place during exercise. This stimulus may act, for example, on the liver fat and thus the oxidation may not be supplying energy directly for muscular contraction. Hence the

exact analyses of this problem must await the determination of acetone bodies in isolated muscles with low carbohydrate supply before and after contraction.

SUMMARY

A study was made of the total acetone bodies in the blood of three subjects before and after exercise with the subjects on normal and low carbohydrate diets. No significant changes were observed following exercise with the subjects on normal diets. On low carbohydrate diets, with a general elevation of the acetone bodies, a further increase was found after work. The maximal rise in each case was observed two hours after exercise. These experiments suggest that fat is used as such to supply the energy for muscular contraction in man.

The author wishes to thank Dr. R. Loucks and Mr. J. Clark for acting as subjects in this investigation.

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A COMPARISON OF THE NORMAL BLOOD PICTURE OF RATS OF TWO DIFFERENT COLONIES REARED UPON DIFFERENT STOCK RATIONS

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For a number of years the study of the reticulocytes of the blood has afforded a valuable index of therapy in both primary and secondary anemias. We have recognized for some time (Fitz-Hugh, Robson and Drabkin, 1933) that the reticulocyte counts upon the normal stock rats of our colony are higher than those reported by other investigators (Beard, Baker and Myers, 1931–32; Stucky and Brand, 1933). Differences in the normal blood picture of stock rats used in various laboratories may be an index of definite physiological differences in the animals themselves. If such variations exist, they may have a specific bearing upon the interpretation of therapeutic results in nutritional anemia investigations, and may prove of more general interest in other problems where the existence of differences in animals, so highly inbred as the white rat, is rarely considered.

With respect to the determination of reticulocytes, it is important to point out that, due to the absence of an accepted standard technique and the relatively large magnitude of error in estimations of this type, one is scarcely justified in comparing the meager results in the literature upon this phase of the blood picture. By reticulocyte we mean an erythrocyte which, after staining with the vital stain, brilliant cresyl blue, shows either a reticular network or the remnants of such a structure within the cell. Such cells are thought to be immature erythrocytes, and are commonly estimated as percentages of total red cells.

This is a report upon a comparative study of the blood picture of normal rats from two different colonies. The animals of one colony had been reared upon a commonly used stock ration. This basal diet, like others in the literature, is so constructed as to yield animals which are readily susceptible to the development of the various avitaminoses. The animals of the other colony had been reared upon a mixture of natural foodstuffs.

METHODS. *Rats used.* Forty-two albino rats of the Wistar Experimental Colony and 74 albino rats from our own colony were used in this

work. The rats chosen were of different age groups, so as to cover the span from early age to complete maturity. For the animals of the Wistar Institute we are indebted to the courtesy and coöperation of Prof. H. H. Donaldson. On different days 3 rats of a particular age group, as they became available, were selected from stock and worked on at once. The animals were then sacrificed and autopsied. The rats of our colony, while originally of Wistar stock, have been inbred for eight years in our laboratory under dietary conditions very different from those used at the Wistar Institute. Only those of our animals which had not been bled previously[1] were selected for comparison with the rats of the other colony.

Diets upon which rats were reared. These data are given since they represent the most obvious difference in the two sets of animals. The Wistar Institute animals were raised upon a dietary of mixed natural foodstuffs. During each week, a fish mixture was fed for 3 days, a beef mixture for 3 days and upon the remaining day fresh vegetables were supplied. Suet, cod liver oil, wheat, barley, or beans were mixed with fish or meat, and the whole thickened with corn meal. Fresh whole milk was also supplied. This dietary aims, by varying the mixtures from day to day, to avoid monotony (Greenman and Duhring, 1931). It is recognized that such a dietary is not suitable for the rearing of animals specifically for the study of the various vitamin deficiency diseases. On the other hand, our own rats were raised upon a slightly modified Steenbock stock diet (1923), especially suitable for rearing animals to be used in vitamin deficiency studies. The composition of this ration, which all the animals we have used received, was: yellow corn, 71 per cent, linseed oil meal, 16 per cent, crude casein, 5 per cent, ground alfalfa, 2 per cent, milk powder, 5 per cent, NaCl, 0.5 per cent, and $CaCO_3$, 0.5 per cent. Fresh whole milk *ad libitum*, as recommended by Steenbock, was not supplied. Great differences thus existed in the nutritional histories of the two groups of rats.

Determination of blood constituents and hemoglobin. The technique used in the various determinations was identical with that described in another publication (Fitz-Hugh, Robson and Drabkin, 1933). On all rats, besides the determinations reported, white blood cell and white blood cell differential counts were made. These studies were carried out as additional evidence to the autopsy findings to insure that the animals were free of acute infections, such as pneumonia, and will not be presented here.

Bartonella muris infection. The Wistar Institute rats are known to be free of Bartonella muris, but infestation with this parasite has been thought to be widespread in other rat colonies. In the acute form of the infection anemia is the main feature, and one group of workers has found Cu and Fe to be protective against this anemia (Marmoston-Gottesman

[1] Unpublished data in our possession indicated that periodic bleeding of the animals had a slight but definite effect upon the hemoglobin and reticulocytes

TABLE 1

AGE, DAYS	NUMBER OF RATS	SEX		WEIGHT, GRAMS			HEMOGLOBIN, GRAMS PER 100 CC.			RED BLOOD CELLS, MILLIONS PER C.MM.			RETICULOCYTES, PER CENT			COLONY
		♂	♀	Average	Low	High	Average	Low	High	Average	Low	High	Average	Low	High	
3	3	2	1	5	4.5	6.5	13.1*			2.5*			28	25	31	Wistar
4	2	2		10	10	10	15.9	15.4	16.3	2.6	2.3	2.8	53	51	55	Wistar
15	3	3		21	21	21	10.2	10.1	10.3	4.8	4.7	4.9	19	14	24	Wistar
20	3		3	29	22	29	9.7	9.4	10.1	4.6	4.5	4.8	13	9	17	Wistar
24	3	2	1	42	39	44	7.7	6.7	8.5	5.2	4.9	5.6	27	24	30	Wistar
30	3	1	2	51	49	54	10.8	9.4	11.6	5.6	4.9	6.0	19	17	20	Wistar
45	3	2	1	106	94	121	13.7	13.0	14.2	6.5	6.2	6.8	8	4	12	Wistar
55	3	1	2	133	125	147	14.5	14.2	14.8	7.4	7.0	7.7	5	5	5	Wistar
65	3		3	156	153	159	18.2	16.6	19.7	8.7	8.5	8.8	5	5	6	Wistar
70	3	3		202	186	224	17.0	16.6	17.5	8.8	8.8	8.9	7	3	10	Wistar
80	3	2	1	176	156	195	17.5	16.9	18.5	9.4	9.2	9.9	2	2	3	Wistar
100	3	3		235	224	243	18.5	18.1	18.8	9.8	9.5	10.2	2	2	3	Wistar
140	3	3		281	244	300	20.0	17.9	20.8	9.5	8.6	10.0	2	1	3	Wistar
180	3		3	207	182	227	18.4	18.1	18.9	8.5	8.3	8.9	3	3	3	Wistar
2	2	2		4.5*			13.4*			2.6	2.2	3.0	94*			Pennsylvania
3	2	2		4.5	4.5	5	12.1*			2.7	2.7	2.8	90*			Pennsylvania
4	1	1		7			12.0			3.0			87			Pennsylvania
6	2	1	1	10	10	10	9.4	8.5	10.3	3.3*			47	43	51	Pennsylvania
8	7	4	3	12	10	12	9.0	8.5	9.9	3.5	2.6	3.9	38	30	50	Pennsylvania
10	2	2		14*			8.7*			4.0	3.9	4.1	41	40	43	Pennsylvania
15	3	3		20	18	22	7.6	6.8	8.9	4.4	4.3	4.7	35	31	40	Pennsylvania
23	2	2		30	28	32	6.4	6.1	6.7	4.6	4.4	4.8	25	23	26	Pennsylaniva
25	7	1	6	32	21	38	11.5	10.9	12.1	6.0	5.2	6.9	16	12	23	Pennsylvania
27†	4	2	2	41	36	47	11.9	9.8	13.6	6.2	6.0	6.6	22	15	32	Pennsylvania
30	5	3	2	47	38	65	13.0	12.3	13.9	7.1	6.6	7.5	14	10	20	Pennsylvania
30††	5	4	1	51	31	63	13.0	11.5	13.9	7.2	6.5	7.9	16	12	20	Pennsylvania
35	6	3	3	66	60	74	13.1	9.8	15.5	7.3	6.0	8.5	17	6	26	Pennsylvania
47	3	2	1	88	81	95	12.9	12.7	13.0	7.3	7.2	7.6	12	10	14	Pennsylvania
59	4	2	2	108	85	140	13.2	12.0	13.8	7.2	6.1	8.0	11	9	15	Pennsylvania
90	5	4	1	166	145	180	14.8	14.5	15.3	8.1	7.8	8.5	8	5	9	Pennsylvania
105	4	2	2	179	152	200	16.8	14.5	18.5	9.2	8.1	10.2	5	3	7	Pennsylvania
115	2	2		207	205	210	17.0	16.9	17.0	8.8	8.5	9.2	3	3	4	Pennsylvania
140	4	3	1	226	184	245	16.6	14.5	17.5	8.1	7.9	8.4	6	4	7	Pennsylvania
180	2	2		262	260	265	15.9	14.8	17.0	7.5	7.2	7.8	9	9	9	Pennsylvania
200	2	1	1	249	220	278	15.3	14.6	16.1	7.6	7.4	7.8	10	9	10	Pennsylvania

* This determination upon 1 rat only.

† Only in this instance did the females have lower hemoglobin and red blood cells, and higher reticulocyte counts, so that it was considered justifiable to average both sexes together. Most females, however, were lower in weight than males of corresponding age.

†† About 6 months later upon another group of rats 30 days old.

and Perla, 1932). It was therefore considered worth while to test whether the rats of our colony harbored this parasite. This was done in a number of animals by the accepted technique of periodic examination of the blood for evidence of anemia after splenectomy[2] and a quest for Bartonella muris in appropriately stained blood smears. These experiments, which need not be presented here, indicated that the rats of our colony were free of the parasite.

RESULTS. The table is practically self-explanatory.[3] The adult rats of the Wistar Institute had definitely lower reticulocyte counts and higher hemoglobin levels than the adult rats from our own colony. The red blood cell counts were also consistently somewhat higher in the adult Wistar stock. If one would calculate the results of the hemoglobin determinations upon a weight basis, the discrepancy would be even more pronounced, for the Wistar animals ran somewhat heavier than ours. During the first 30 days of life, similar interesting transitional changes in hemoglobin, red blood cells and reticulocytes took place in both the Wistar and Pennsylvania animals. During this early period only the reticulocytes were markedly discrepant, the Pennsylvania animals showing very high percentages. At 23 days of age each group of rats was at the peak of transitional anemia, our rats having been slightly more anemic than the Wistar. At this time, which corresponded to the weaning period, in both groups of animals there was a relatively sudden break in the hemoglobin curve in the direction of fairly rapid increase to adult levels. Thus, the age at which rats are transferred to milk, whether it be 23, 25 or 27 days, may have some influence upon the course of the anemia. The decrease in hemoglobin from birth to weaning has not been noted by Mitchell and Miller (1931) in their studies, although the level of hemoglobin of their adult animals was similar to that of ours. In particular age groups, the rats of the Pennsylvania colony showed a greater spread in hemoglobin and red blood cell determinations than the Wistar animals of corresponding age. However, even in our colony averaging of figures was thought to be permissible, as may be seen by the remarkably close agreement of the various determinations upon two sets of 30 day old rats, studied about six months apart.

[2] Kindly performed for us by Dr. I. S. Ravdin of the Department of Research Surgery.

[3] In order to present our data adequately, they are given according to the exact age in days of the animals. While the number of animals used may be insufficient to establish the exact values of hemoglobin, etc., for a particular age, an analysis of the data upon the Wistar and Pennsylvania rats indicates that a sufficient number of animals has been used to establish the trend of the various determinations as related to age. This becomes more apparent when the animals are taken in larger groups, as those from 3 to 24 days, 30 to 60 days, etc.

SUMMARY AND CONCLUSIONS

The blood pictures of rats of various age groups from two different colonies have been studied. The interesting transitional changes from birth to weaning may be said to be established since parallel phenomena were observed in the bloods of rats from the two colonies. Definite differences in the blood pictures of the mature animals of the two colonies have been shown to exist.

These differences in hemoglobin level and reticulocytosis may be related to the markedly different nutritional backgrounds of the animals. Such differences in the animals used by various investigators of the problem of nutritional anemia may be among the factors responsible for the discrepant results in the literature.

From the standpoint of other nutritional investigations, besides those of anemia, it is considered worth while to call attention to the existence of physiological differences in animals so highly inbred as the albino rat.

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THE PROPORTION OF RETICULOCYTES IN THE BLOOD OF ALBINO RATS[1]

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Recent investigators in the field of experimental hematology have made increasing use of the albino rat. As a result, numerous data have accumulated in the literature regarding both the normal values for certain of the blood constituents in this species and deviations due to a variety of experimental conditions. In the majority of these studies, special stress has been placed on the determination of the erythrocyte and hemoglobin values; normal variations in the concentration of these constituents are therefore known with some degree of certainty. The present knowledge of certain other blood values, however, is far from complete. Particularly is this true of the vital-staining erythrocyte, the reticulocyte. Relatively few comprehensive studies of the proportion of these cells in the blood of rats have appeared in the literature. In view of this fact and of the recently demonstrated importance of the reticulocyte as a clinical index of hematopoietic activity, further information regarding the variations of the reticulated erythrocytes in the blood of the rat seems urgently needed. This report presents data on the proportion of reticulocytes together with simultaneous determinations of the hemoglobin concentration and erythrocytes in the blood of normal rats of different ages.

EXPERIMENTAL. *Care and selection of animals.* Albino rats of the Connecticut Agricultural Experiment Station strain were used. With the possible exception of the very young animals, newborn to 10 days of age, only males were employed. The animals in each of the age groups between the "newborn" and "21-day old" limits were housed in litter groups with their mothers and were allowed access to the stock ration described below. From the time of weaning, 21 days of age, the 25 and 30-day old rats likewise were kept in litter groups and were fed the stock colony ration. In all instances, the data given for animals from birth to 30-day limits were

[1] A preliminary report was made before the Biological Section of the American Chemical Society at the Chicago Meeting, September, 1933. Aided by a grant from the Research Funds, Yale University, School of Medicine.

[2] Alexander Brown Coxe Fellow, Yale University, 1932–33.

obtained from different individuals, inasmuch as there exists the possibility that, because of the small size of animals of these ages, the removal of even the small amount of blood necessary for reticulocyte determinations might itself result in an alteration of subsequent counts. Two groups of 12 rats each were studied at age intervals beyond 30 days. These animals weighed 40 to 50 grams at weaning, at which time they were placed in individual cages, cared for in the routine manner described by Smith, Cowgill and Croll (1925), and given the stock diet. When the animals reached 37 days of age, one group of 12 was continued on the stock ration and an equal number was changed to the "adequate synthetic" diet. At this time, also, routine blood determinations were begun at the intervals shown in the tables. When the animals of both groups reached an age of 177 days, the routine blood determinations were suspended for a period of 28 days before the final data were obtained. The purpose of this "rest" period was to allow an interval for an undisturbed adjustment of the blood constituents to "normal" levels, in case there had occurred an alteration as a result of the previous routine withdrawal of samples.

Diets. The stock colony ration consisted of calf meal,[3] in which was incorporated 3 per cent cod liver oil, fed *ad libitum*, approximately 10 grams of fresh lettuce twice weekly, and 1 gram of dried yeast three times weekly. During the first three weeks after weaning, this ration was supplemented by a paste food containing whole milk powder 25 per cent, casein 25 per cent, wheat embryo 20 per cent, and lard 30 per cent.

The "adequate synthetic" diet contained specially washed casein (Swanson, 1930) 18 per cent, dextrin 51 per cent, Crisco 27 per cent, and Osborne and Mendel (1917) salt mixture 4 per cent. The daily vitamin supplements consisted of 100 to 120 mgm. of cod liver oil, 200 mgm. of dried yeast, a concentrate of an alcoholic extract of wheat germ equivalent to 2 grams of embryo, and 40 to 50 mgm. of wheat germ oil. The method of preparation of the latter two supplements has been described by Swanson (1930).

Methods. At the intervals indicated in the tables, hemoglobin, erythrocyte and reticulocyte determinations were made on freely-flowing tail blood. Samples were obtained from the rats less than 37 days of age by clipping the end of the tail and from the older animals from a small incision into one of the tail vessels. Extreme care was exercised to prevent undue loss of blood. Hemoglobin determinations were made by the acid-hematin colorimetric technique (Cohen and Smith, 1919), using a standard glass disc calibrated by the oxygen-capacity method. A constant source of artificial light in a darkened room was used for illumination and the position of the colorimeter was carefully adjusted before readings were taken. Erythrocyte enumerations were made in duplicate. The routine reticulo-

[3] For composition, see L. A. Maynard, Science, 1930, **71**, 192.

cyte determinations were performed on "permanent" smears prepared by the Cunningham (1920) technique. In addition to the use of chemically clean apparatus and properly made stains, careful attention to several technical details was found essential for the preparation of satisfactory smears: 1, the preparation of pairs of stained cover slips with inner surfaces completely covered by a uniform smooth coat of brilliant cresyl blue; 2, the prompt manipulation in obtaining and in placing the sample of blood in contact with the stained cover slips in order to minimize initial clotting; 3, the use of a drop of blood of such size that the film formed after spreading just filled the space between the diagonally-crossed cover slips; 4, the preparation of a thin uniform film in which the cells were clearly separated by the aid of gentle pressure on the cover slips with the thumb and forefinger and by the even pulling of the slips. The pairs of slips showing satisfactory films were dried, counterstained with Wright's, and mounted with balsam. The mounted slides have been kept satisfactorily for as long as 12 months. The percentage of reticulated cells was determined by the examination of at least 500 cells on each of the pair of films, using oil immersion magnification. A field constricting disc[4] was placed in the oculars of the binocular microscope to limit to five or six the number of cells appearing in the field at any one time. The total red cells observed were tallied on the usual erythrocyte counter and the reticulated cells on another. The foregoing technique has proved entirely satisfactory for routine determinations, not only because one obtains a permanent record of the reticulocyte picture which may be referred to at any time, but also because only a small amount of blood is required, thus minimizing the danger of an alteration of the proportion of reticulated cells from an excessive loss of blood (Seyfarth, 1927).

Inasmuch as there may occur a decolorization of some of the finer grades of reticulocytes during the process of counter-staining in the "permanent" technique (Seyfarth, 1927), resulting in a slightly low count, repeated check reticulocyte counts were made on a number of the rats using non-counterstained specimens, the so-called "wet" preparations. Two different methods for making "wet" preparations were used. In one, the same procedure was employed as in the "permanent" technique previously described except for the omission of the counter-stain. The thin film of cells between the stained surfaces of the cover slips was protected from evaporation by vaseline applied to the coinciding edges. This type of preparation must be counted promptly inasmuch as there may occur upon standing a migration of the reticulocytes to the periphery which, of course, would vitiate the results. The other "wet" method used was a modifica-

[4] May be cut from a hard grade paper or from heavy tin foil. The paper disc of proper size should be folded into fourths and a triangle having about a 2 mm. base cut from the apex.

tion of the Osgood and Wilhelm (1931) procedure. The sample of blood was drawn up to the "4" or "5" mark in a leucocyte diluting pipette and an equal volume of a 1 per cent solution of brilliant cresyl blue in 0.85 per cent sodium chloride was added. The entire column was then carefully drawn up into the mixing chamber of the pipette and thoroughly shaken. After staining for at least 2 to 3 minutes, the pipette was again thoroughly shaken and a drop of the mixture of such size was discharged onto a clean cover slip so that, when a second cover slip was placed on the first, a uniform thin film of cells filled the entire space between the cover slips. The preparation was then sealed with vaseline, placed on a slide, and promptly counted under oil immersion.[5]

RESULTS. The data obtained in this study are given in the accompanying tables. The average body weights of the stock rats (table 1) fall near the upper limits of those previously reported (Smith and Bing, 1928) for this colony. The hemoglobin and erythrocyte levels showed a progressive parallel increase from the initial values to adult levels having an average variation of from 15.1 to 16.4 grams per 100 cc. and from 8.5 to 9.2 millions per cubic millimeter, respectively. The terminal values, obtained after the 28 day "rest" period, agreed well with the preceding values, which indicates, we believe, that the amount of blood removed during the routine analyses on these animals was not of sufficient magnitude to affect the previous data. These hemoglobin and erythrocyte data for stock rats are in general accord with other similar data in the literature (Happ, 1922; those referred to by Scarborough, 1930–31; Orten, Underhill, Mugrage and Lewis, 1932; C. Smith, 1932; and Stucky and Brand, 1933). The change in pigment concentration with age agrees with that obtained by the spectrophotometric technique used by Williamson and Ets (1926). The normal reticulocyte variations with age are of special interest. In the blood of the newborn rats there existed a remarkably high proportion of reticulated cells, average 98.7 per cent. This value agrees well with that obtained on femoral blood by Happ (1922) and on heart blood by Smith (1932) but is higher than that described by Seyfarth (1927) and by Stucky and Brand. The initially high reticulocyte count decreased rapidly until at the weaning age, 21 days, only 25 per cent of the erythrocytes exhibited the vitally staining material. When the animals had reached 70 days of age, the reticulocytes had decreased to a level of about 3 per cent. During the remainder of the experiment there occurred an average variation of the reticulocytes between limits of 1.7 and 2.8 per cent. Using "permanent"

[5] Permanent preparations made by spreading, drying, and counter-staining samples stained by this method did not yield satisfactory results, possibly because during the drying process, even though rapidly done, a shrinkage of the cells often occurred and lower counts were obtained than on the non-counter-stained preparations or on smears made at the same time by the Cunningham technique.

methods of reticulocyte enumeration, similar values for adult rats have been reported by several other investigators (see Happ, 1922; Vaughan and Gunn, 1929–30; Vaughan and Muller, 1932; Ma, 1932; Smith, 1932; and Stucky and Brand, 1933).

TABLE 1

*Hemoglobin, erythrocytes, and reticulocytes in the blood of normal stock rats**

DAYS OF AGE	NUMBER OF RATS	BODY WEIGHT, GRAMS		HEMOGLOBIN, GRAMS PER 100 CC.		ERYTHROCYTES, M. PER C.MM.		RETICULOCYTES, PER CENT	
		Group average	Limits of variation	Group average	Limits of variation	Group average	Limits of variation	Group average	Limits of variation
0	6	6	5– 7					98.7	98.0–99.4
5	5	10	9– 11					62.8	58.8–69.0
10	5	16	16– 17					44.0	37.0–51.0
15	5	21	20– 22					24.3	20.3–28.4
20–22	6	42	33– 51					25.8	20.2–34.7
25	5	49	40– 58					14.4	11.3–16.1
30	6	66	57– 75					13.1	10.3–16.1
37	12	111	94–124	10.7	9.2–13.3	6.4	5.6–8.1	8.9	6.5–14.6
44	12	149	121–170					6.9	3.6–12.8
51	12	182	156–207	13.7	12.3–15.8	7.0	5.8–8.0	4.5	3.2– 6.6
58	12	212	180–242					4.1	2.0– 5.3
65	12	247	214–293	14.2	11.9–16.2	7.8	6.3–9.3	3.2	2.8– 4.5
72	12	267	236–331					2.8	1.2– 4.5
79	12	289	246–364	15.2	13.4–16.4	8.0	6.9–9.4	2.5	0.8– 3.5
86	12	309	274–388					2.0	1.3– 2.6
93	12	335	292–420	15.2	14.4–15.7	8.1	7.2–9.3	2.2	1.6– 2.8
100	12	353	309–434					2.5	1.7– 3.2
107	12	368	325–452	15.2	14.2–15.6	8.9	8.6–9.6	2.8	1.4– 4.6
114	12	383	338–470					2.0	0.8– 3.6
121	12	390	343–472	16.0	15.5–16.4	8.7	8.1–9.1	2.8	1.0– 4.5
128	12	399	352–471					2.3	1.0– 3.2
135	12	414	354–486	15.6	14.3–16.6	8.6	7.2–9.7	1.9	1.2– 2.4
142	12	423	361–510					2.1	1.2– 3.4
149	12	431	369–535	15.7	14.3–16.8	8.8	8.1–9.4	1.9	1.0– 2.7
156	12	439	375–530					2.0	1.0– 3.1
163	12	449	384–541	15.8	13.7–16.9	8.9	8.3–9.7	2.3	1.4– 3.2
170	12	452	368–544						
177	12	477	424–557	15.5	14.9–16.2	9.4	7.8–9.7	1.7	1.2– 2.4
205	12	486	424–586	15.7	14.0–16.9	8.6	8.1–9.6	2.4	1.7– 3.4

* With possible exception of those under 15 days of age, only males were used.

In the relatively small number of cases studied, slightly higher reticulocyte counts were obtained on preparations made by either of the "wet" techniques, previously described, than on "permanent" smears prepared at the same time. Values for normal adult rats ranged from 1.1 to 5 per cent. In all cases, the magnitude of the counts obtained by the two types

of technique showed parallel fluctuations. Reticulocyte variations on "wet" preparations between the limits of 1.0 and 5.1 per cent have been found by Adams and Shevket (1929) in a limited number of observations; similar values have been reported by Seyfarth (1927) and by Beard, Baker and Myers (1931). The stock rats used by the latter group of investigators, however, were younger than those which we have found to give re-

TABLE 2

Hemoglobin, erythrocytes, and reticulocytes in the blood of normal male rats fed an "adequate synthetic" ration

DAYS OF AGE	NUMBER OF RATS	BODY WEIGHT, GRAMS		HEMOGLOBIN, GRAMS PER 100 CC.		ERYTHROCYTES, M. PER C.MM.		RETICULOCYTES, PER CENT	
		Group average	Limits of variation	Group average	Limits of variation	Group average	Limits of variation	Group average	Limits of variation
37	12	118	116–121	11.5	9.2–14.3	6.4	5.6–7.8	7.2	4.9–9.3
44	12	145	130–157					4.8	2.2–9.8
51	12	173	146–191	13.7	11.4–14.9	7.9	6.7–9.3	3.5	1.6–4.4
58	12	197	176–220					2.8	1.5–4.5
65	12	219	200–244	14.8	13.3–15.8	8.5	7.1–9.4	2.6	1.7–4.1
72	12	240	218–261					2.1	1.3–3.3
79	12	255	236–274	15.8	14.3–19.1	8.9	7.8–9.6	1.9	0.8–4.3
86	12	273	250–297					2.0	1.0–3.1
93	12	285	260–308	15.3	14.1–16.9	8.9	8.0–9.9	1.9	1.0–2.9
100	12	304	275–326					1.5	0.5–2.2
107	12	316	280–337	15.1	12.2–16.3	9.0	7.5–10.8	2.6	1.7–6.2
114	12	332	294–360					2.6	1.1–4.9
121	12	343	306–364	15.5	14.5–16.4	8.7	8.1–9.7	2.0	0.9–4.1
128	12	347	310–376					1.8	1.1–3.7
135	11	350	318–380	16.1	14.1–18.6	8.9	8.1–9.9	1.7	0.8–3.0
142	11	359	320–384					1.7	0.8–3.3
149	11	361	324–392	16.0	14.8–18.0	8.9	8.3–10.1	1.8	1.0–2.5
156	11	371	326–397					2.0	1.0–3.6
163	11	374	330–410	15.9	14.3–17.4	8.9	8.2–9.6	1.7	0.8–3.6
170	11	376	327–408					1.8	0.9–2.8
177	11	378	334–420	16.4	15.2–18.3	9.1	8.6–10.1	1.6	0.8–2.9
205	11	398	341–437	16.0	14.4–18.0	9.2	8.1–9.8	1.6	1.2–2.1

sults within the adult range. Values higher than 5 per cent have been obtained on "wet" preparations on certain adult stock rats by Drabkin.[6]

The data in table 2 were obtained from the group of rats consuming the "adequate synthetic" diet. These results are in general agreement with those obtained on the stock rats of comparable ages. There occurs the same tendency for the hemoglobin and erythrocyte values to increase and the reticulocyte level to decrease to adult limits similar to those of the stock group. However, it may be noted that the changes from the ju-

[6] Personal communication.

venile to the characteristic adult values occur somewhat more promptly in the animals administered the "adequate synthetic" ration than in the stock rats. This may be due to the fact that the animals employed in the former group were slightly heavier at the beginning of the experiment and hence exhibited a somewhat more "mature" blood picture than the stock rats of the same age.

The same fluctuations of reticulocyte values with age have been observed in animals given a milk-iron-copper diet. Adult rats, weighing from 320 to 510 grams, which have consumed this mixture since weaning have shown reticulated cells varying between 1 and 4 per cent.

Throughout these experiments there has existed a remarkably sensitive relation between the concentration of pigment and total red cells and the proportion of reticulocytes. Under certain conditions when there has occurred a significant decrease in hemoglobin and erythrocytes, an increase in the reticulocyte count was observed. A skin infection which resulted in a decrease in hemoglobin and red cells from 15.8 grams per 100 cc. and 9.3 million per cubic millimeter to 12.9 grams and 7.2 million, respectively, was accompanied by a sharp increase in the reticulocytes from 2.5 per cent to 6.2 per cent. An anemia (Bartonella?) in which the hemoglobin and erythrocytes decreased to 5.0 grams per 100 cc. and 1.9 million per cubic millimeter, respectively, in several of a group of splenectomized rats was accompanied by a pronounced reticulocytosis, the values attaining a maximum of 40 per cent. These findings emphasize the necessity of extreme care in preventing infections in rats used for studies in which the proportion of reticulocytes is under investigation.

SUMMARY

The blood of normal albino rats at birth shows a remarkably high proportion of reticulocytes (99 per cent). The percentage of reticulated cells decreases rapidly after birth until at the weaning age (21 days) only 25 per cent of the cells show the vital-staining material. Thereafter, a more gradual progressive decrease in the reticulocyte level occurs until at about 70 days of age an adult value, varying between 1 and 3 per cent is reached. The blood of rats maintained on an "adequate synthetic" diet from an initial age of 37 days shows similar changes in the reticulocyte level as those found in stock rats.

There exists a remarkably sensitive relation between the concentration of blood pigment and total red cells and the proportion of reticulocytes in the circulating blood of the albino rat.

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AN X-RAY DIFFRACTION STUDY OF THE EFFECT OF RACHITIS UPON THE STRUCTURAL CHARACTERISTICS OF BONE

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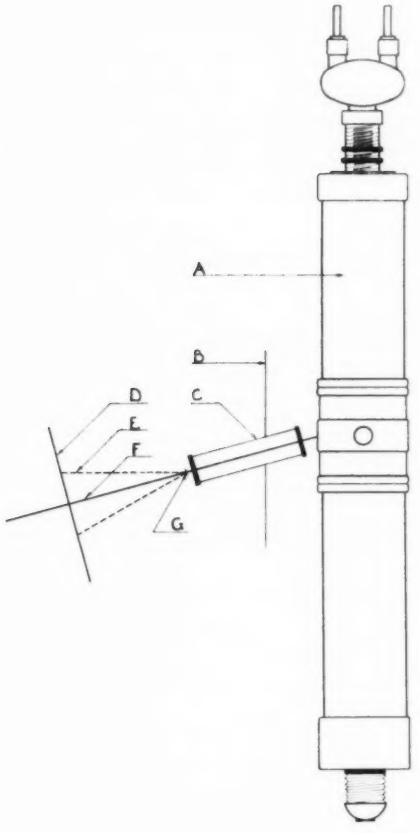
HISTORICAL. A vast amount of data has been reported in the literature upon the *composition* of bone and the effect of various abnormal physiological conditions thereon, but, strange to say, very little work has been concerned with the effect of these abnormal conditions upon the *structure* of bone.

Only in recent years have attempts been made to apply x-ray diffraction methods to an investigation of bone, tissue, cartilage, and other physiological systems. Although some references in the literature are concerned with diffraction studies of normal and pathological tissue, most of the x-ray work has been confined to an investigation of normal bone. This work has progressed to such an extent that at the present time the structure of normal bone is fairly well understood.

Roseberry, Hastings and Morse (1), using the powder diffraction technique on the familiar General Electric molybdenum apparatus, agreed with previous investigators—Hendricks, Hill, Jacob, and Jefferson (2), deJong (3), and Bassett (4)—concerning the essential similarity between bone and the carbonate-substituted apatite, dahlite, $CaCO_3 \cdot n\ Ca_3(PO_4)_2$, where n is not less than 2 and not greater than 3. Moreover, Roseberry, Hastings, and Morse concluded that the crystal structure of bone is one belonging to the close-packed hexagonal lattice, containing four molecules per unit cell of dimensions a = 20.8 A.U., b = 12.0 A.U., and c = 8.82 A.U. deJong reports that inorganic crystals of bone contain only a few hundred molecules and sometimes less.

TECHNIQUE EMPLOYED AND THE RESULTS OBTAINED THEREWITH. In this investigation we have considered the effect of rachitis upon the structural characteristics of bone using x-ray diffraction methods. Figure 1 is a schematic representation of the experimental conditions, the monochromatic pinhole technique being employed throughout the investigation. An optimum specimen thickness of approximately 0.15 mm. was selected for all the patterns.

Figure 2 is the pattern obtained with the humerus of a normal rat. It



A = Muller Kreuzfok Tube (Cu)
B = Lead Shield
C = Pinhole System
D = Plate
E = Diffracted Ray
F = Central Ray
G = Specimen
2.5ma · 30Kvp · 2hrs
Plate-Specimen = 3 cm

Fig. 1

Fig. 2

Fig. 3

will be noticed that the inner diffraction ring is the only one that is sharply arced. Since arcing in diffraction patterns such as these is characteristic of a preferred orientation, and since this arcing in normal bone is parallel to the longitudinal axis of the bone, we are led to the conclusion that the inorganic crystals of bone are oriented lengthwise along the bone. The sharp diffraction rings of figure 2 are susceptible to accurate measurement and, by means of the Bragg equation, to an evaluation of atomic spacings. Table 1 represents a compilation of the spacings in Angstrom units obtained by Roseberry, Hastings, and Morse (1), Janet Clark (5), and by this research on normal bone.

TABLE 1

ROSEBERRY, HASTINGS, AND MORSE	JANET CLARK	THIS RESEARCH
3.35	3.40	3.38
3.02		3.07
2.72	2.82	2.70
2.25	2.25	2.25
1.93		1.92
1.83		1.81

The agreement between the three different sets of values is quite satisfactory.

Figure 3 is the pattern obtained with a rachitic bone. Two prominent differences between the normal and rachitic patterns are immediately noted: 1, the degree of arcing of the inner diffraction ring has lessened appreciably for the rachitic bone, indicating a loss of preferred orientation along the long bone axis, and 2, the diameter of the innermost ring indicative of amorphous material has increased from a maximum value of 2.05 cm. for normal bone (corresponding to a spacing of 4.67 A.U. when calculated from the equation $n\lambda = 2d\sin\theta$) to a value of 2.70 cm. (3.67 A.U.) indicating that the organic constituent of bone has been broken down under the influence of rachitis. Since no change was found in the spacings calculated from the diameters of the sharp diffraction rings, it follows that no change has taken place in the chemical composition of the inorganic material of the bone.

The results obtained on a series of nine bones of varying degrees of rickets are summarized in table 2. The data under "Description of Bone" were obtained in the usual radiographic manner by Dr. C. I. Reed of the University of Illinois College of Medicine.

The diameter of the amorphous ring is a function of the severity of rickets, being in the neighborhood of 2.0 cm. for normal bone and 2.7 for the severely rachitic bone. On this basis the diagnosis of rickets by the diameter of the amorphous ring agrees with the radiographic diagnosis in

all but two cases, bone no. 5 being found completely healed instead of well healed and bone no. 7 being found partially instead of completely healed. The slight disagreement may readily be understood if one considers the difficulty of quantitatively diagnosing rickets by the radiographic method. The relative constancy of the diameters of the diffraction rings indicated that inorganic material does not change its crystal structure under the influence of rickets.

In addition to the above investigation on rachitic bones, the work of Janet Clark on the longitudinal orientation of the inorganic micelles of normal bone was completely verified. This work was repeated because it was found in the literature that Roseberry, Hastings, and Morse were at variance with Miss Clark's results. A possible explanation of this disagreement may be advanced for it was found that although the bone is

TABLE 2

DESCRIPTION OF BONE	DIAMETERS IN CENTIMETER				
	Amorphous band	1st ring	2nd	3rd	4th
Normal	2 08	2 99 (*f*)	3 40	3 92	5 01
Severe rickets	2 70	3 00 (*p*)	3 43	3 92	5 16
Slight healing	2 20	3 00 (*pp*)	3 32	3 89	5 03
Good healing	2 01	3 00 (*n*)	3 35	3 92	5 01
Complete healing	2 06	3 00 (*n*)	3 39	3 92	5 12
Complete healing	2 23	3 00 (*n*)	3 42	3 90	5 09
Complete healing	2 06	2 96 (*ppp*)	3 32	3 89	5 12
Partial healing	2 29	3 01 (*ppp*)	3 38	3 89	5 05
Complete healing	2 00	3 00 (*ppp*)	3 36	3 87	5 08

f equals sharp arcing, *p* equals small amount of arcing, *pp* equals traces of arcing *n* equals no noticeable arcing, *ppp* equals faint traces of arcing.

fibered along most of its surface the fibrous structure was lost within that region where a process was attached to the main bone.

Diffraction photographs were taken with the x-ray beam perpendicular to the long axis of the bone, one in a north-south direction and the other in an east-west direction, both showing fibering. Another pattern was taken with the x-ray beam parallel to the long axis and shows no fibering, the diffraction ring corresponding to the longest spacing being practically invisible. These results are consistent with the view that the micelles are oriented longitudinally.

Discussion of results. The above results can be interpreted by considering that the attack of rachitis upon the bone is a breaking down of the organic "cement" (collagen or ossein) which holds the inorganic crystals in their normal oriented position. After this "cement" has been de-

composed, the inorganic material, although remaining chemically unchanged, loses its preferred orientation and the bone loses its resistance to strain in much the same manner as a tied bundle of sticks loses its supporting strength when untied and scattered. Moreover, since the healed rachitic bone yields patterns which show but a small amount of fibering, the conclusion follows that once the crystallites of the normal bone have lost their preferred orientation, due to the effect of rickets, they do not regain their ordered arrangement once the bone becomes healed, even though the organic material does resume again its normal form. This interpretation is in harmony with the observation by Klement (6) based upon extraction experiments that the organic salts of bone are not chemically combined with the inorganic constituents.

A considerable amount of work has been done in recent years with a view to clarifying the present concepts concerning the equilibrium thought to exist between the calcium in the serum and the calcium of the bone, and several theories have been advanced concerning this equilibrium—Shear and Kramer (7), Holt, LaMer and Chown (8), Freudenberg and Gyorgy (9), and Robinson and Kay (10). It is seen from the results presented above that the mechanism of operation of this equilibrium must consider, not only the serum calcium-bone calcium equilibrium but also the serum-organic bone material equilibrium, for the latter equilibrium must have a profound effect upon the ease with which the bone calcium is dissolved or precipitated.

CONCLUSIONS

1. The longitudinal orientation of the inorganic micelles of bone has been confirmed.

2. Rachitis destroys this preferred orientation to a large extent.

3. Upon healing, the inorganic micelles of a rachitic bone do not regain their preferred orientation although the organic constituent assumes the same form as in the normal bone.

4. The effect of rachitis upon normal bone may be considered as a breakdown of the organic "cement" which holds the inorganic micelles in their oriented position.

5. A new method of quantitatively diagnosing the severity of rickets has been developed.

6. It has been shown that any theory involving equilibria between serum calcium and bone calcium must take into account the more fundamental equilibria between serum and the organic constituent of bone.

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THE EFFECT OF CYANIDE AND OTHER SUBSTANCES ON THE OXYGEN UPTAKE OF RAT TISSUE

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According to Warburg's theory of cell respiration, the oxidation agent of respiration is molecular oxygen and the primary reaction is between molecular oxygen and iron (1921–1923a). This conclusion was the result of a study of a number of systems in which the addition of cyanide inhibited oxidation while the addition of minute quantities of iron markedly catalyzed certain reactions. Dixon and Elliott (1929) have recently made an extensive study of the effect of cyanide on the respiration of various animal tissues. These workers observed that the maximum inhibition varies in different tissues between 40 and 90 per cent, the average value being about 60 per cent. From this it was concluded that Warburg's "respiratory enzyme" (Keilin's cytochrome-indophenol-oxidase system) can therefore account at the most for only about two-thirds of the total respiration of animal tissues.

A study was begun in this laboratory to compare certain tissue reactions (mainly on liver) of normal rats and rats made anemic by being placed solely on a milk diet. It was observed that the indophenol oxidase color reaction employing the "Nadi" reagent was markedly less intense employing liver from severely anemic animals when compared with liver from normal animals. Since cyanide completely inhibits the indophenol color reaction, it seemed interesting to compare the effect of cyanide on the respiration (rate of oxygen uptake) of liver tissue with the degree of indophenol color reaction.

In addition, experiments were performed with rat tissue employing urethane, sodium pyrophosphate and arsenite, both in the presence and absence of para-phenylenediamine, with the object of obtaining further information as to the mode of action of these substances.

METHODS. The method employed to determine the amount of indophenol blue produced by the tissue was essentially that described by Dye (1926) who made use of the "Nadi" reagent. The results are expressed in per cent of total color. The per cent of control color was subtracted from the color produced by the tissues. In all instances the same weight of

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tissue and length of time were employed and consequently the values obtained represent relative amounts of indophenol blue produced. The time interval for the development of the indophenol blue was 30 minutes. The various procedures were performed as uniformly as possible employing animals of the same age. The animals were killed by a blow on the head and the tissue removed immediately so that the estimation of the oxygen taken up by the tissue and the determination of the indophenol blue production could be started as quickly as possible. Enough of the liver tissue was placed in Ringer's solution[2] and carefully sliced preparatory for the estimation of oxygen consumption. The remainder of the tissue was employed to determine the indophenol blue production. The amount of oxygen taken up by the tissue was measured employing Warburg vessels in an atmosphere of O_2 at 38°C.; KOH being placed in the cup of the vessel to absorb the CO_2. The tissue slices were prepared as described by Warburg (1923b). Great care was taken to obtain tissue slices of the same size and thickness since it has been shown that the amount of oxygen taken up by the tissue depends to some extent upon the thickness of the slices (Minami, 1923). The KCN was neutralized before use.

EXPERIMENTAL RESULTS. *Indophenol oxidase color and inhibition of O_2 uptake by M/150 KCN.* Table 1 shows the average rate of oxygen uptake of rat tissue in Ringer's solution containing M/150 KCN compared to the indophenol oxidase color reaction. As is observed the indophenol blue production is on the average definitely less employing liver obtained from anemic animals when compared with normal liver. Further, the ability to produce indophenol blue is rapidly regained when the anemic animals' diet is supplemented with iron or iron and copper. As a matter of fact, values definitely above normal may be obtained when the iron or iron and copper has been given for a period.

Employing rat liver it is observed from table 1 that although the amount of indophenol color produced varied quite widely, both the amount of oxygen uptake and the per cent of oxygen uptake inhibited by the cyanide remained comparatively constant. Further, the inhibition of oxygen uptake of rat kidney, spleen and muscle by the cyanide also shows no relationship to the average amount of indophenol blue produced. For example, spleen with the "Nadi" reagent produces very little indophenol blue when compared with that obtained employing kidney tissue, yet the effect of M/150 cyanide was essentially the same in both instances.

[2] Except where otherwise stated the Ringer's solution employed was that described by Richardson (1929) which is prepared as follows:

NaCl, 9 gm. per liter	1000 cc.
KCl, 11.5 gm. per liter	20 cc.
$CaCl_2$, 12.2 gm. per liter	20 cc.

To 9 cc. is added 1 cc. of M/15 sodium phosphate mixture, pH 7.4.

Keilin (1929) observed that yeast cells contain a powerful indophenol oxidase system which is revealed by the oxidation of the "Nadi" reagent or of paraphenylenediamine. With untreated yeast cells the oxidation of these reagents was observed to be prevented by reducing systems of the cells so that Keilin found that the positive oxidase reaction was obtained only when the activity of these reducing systems was inhibited by *a*, various narcotics such as urethane; *b*, warming the cells for 1½ hours at 52°C.; *c*, lowering the

TABLE 1

Average rate of O_2 uptake of rat tissue in Ringer's solution and Ringer's solution containing M/150 KCN compared to the indophenol oxidase color reaction*

NUMBER OF ANIMALS	O_2 UPTAKE IN MINUTES				O_2 UPTAKE INHIBITED BY CYANIDE		INDOPHENOL BLUE† COLOR REACTION	REMARKS
	Ringer's solution		Ringer's solution + M/150 KCN					
	60	120	60	120	60	120		
	Cu. mm./mgm. dry tissue				*per cent*		*per cent of total color*	
2	5.2	10.3	2.0	6.6	62	36	65	Liver (4 weeks 2.0 mgm. Fe daily)
2	7.6	14.8	4.7	7.4	51	50	61	Liver (4 weeks 0.5 mgm. Fe + 0.05 mgm. Cu daily)
3	9.3	17.3	4.9	7.9	47	54	48	Liver (2 weeks 0.5 mgm. Fe daily)
6	6.1	12.7	3.6	7.4	41	42	41	Liver (normal)
3	8.2	15.7	4.2	7.4	50	53	39	Liver (2 weeks 2.0 mgm. Fe daily)
7	7.3	14.5	3.5	6.6	52	54	10	Liver (anemic)
3	8.8	16.8	4.1	7.7	53	54	6	Liver (1 week 0.5 mgm. Fe daily)
5	12.5	24.1	3.5	6.8	72	72	40	Normal kidney
3	13.5	25.4	3.6	6.6	73	74	25	Anemic kidney
5	12.2	22.4	4.1	6.9	66	69	0–10	Normal spleen
5	3.1	5.5	2.2	3.5	29	36	15	Normal muscle

* Ringer's solution in the case of muscle contained no calcium.

† The indophenol blue values for kidney, spleen and muscle are taken from a series of other animals.

temperature of the cell suspension to −2°C. With the heart muscle suspension Keilin found that the oxidized dye developed on shaking did not reduce to the leuco compound on standing which, he states, shows that the muscle suspension is devoid of reducing substances which are present in the unwashed muscle. Further, he found that while M/1000 KCN or H_2S completely inhibited the indophenol reaction, ethyl urethane (5 per cent) and sodium pyrophosphate (N/30) had no effect on its activity.

Consequently, it was of interest to compare the effect on the amount of

indophenol blue produced when both normal and anemic rat liver tissue was heated to 52° for 1½ hours, and further the effect of the presence of urethane in 5 per cent concentration. Although a number of trials of heating both normal and anemic liver tissue at 52° for 1½ hours were made, suspending the tissue both in Ringer's solution and in M/15 phosphate buffer, in every instance a marked decrease of the indophenol blue color produced was observed when compared with the control. This was a distinct contrast to the suspension of yeast cells which after heating showed, as Keilin pointed out, a marked increase in the indophenol blue production.

The effect of the presence of 5 per cent urethane on the indophenol blue production was observed employing anemic rat liver, normal rat liver, muscle, spleen and kidney. In every instance a definite decrease in the amount of indophenol blue production was observed, although urethane added to the reagents without tissue showed an increase in the amount of color.

Tissues heated to boiling temperature or tissues in the presence of M/1000 KCN showed no indophenol blue reaction.

As has been observed, the "Nadi" reagent gradually undergoes autoxidation when in contact with air. It was shown by Wertheimer (cited by Keilin, 1929) that this autoxidation is due to the presence of traces of heavy metals such as iron and copper. The effect of a number of heavy metals on the autoxidation of this reagent was tried. Of the group copper was found especially active in accelerating the reaction. As small quantities as 0.0005 mgm. copper added to the "Nadi" reagent showed some accelerating action. Iron, cobalt, mercury and vanadium showed some increase in the indophenol blue produced when added to the reagent. The increase in indophenol blue production resulting from the addition of 0.25 mgm. of these metals to the reagent was comparable in a given time to that observed with the addition of 0.0025 mgm. of copper. It was observed that the increase in the amount of indophenol blue produced upon the addition of small amounts of copper to the reagent as copper sulfate was directly proportional in a given time to the amount of copper added. Copper added to the reagent in the presence of tissue or yeast, however, did not show this marked action.

Warburg (1931) has recently criticized the conclusions of Dixon and Elliott (1929), his main objection resting upon the fact that the latter workers suspended their tissues in a phosphate buffer which he states injures the cells. It is Warburg's claim that complete inhibition with cyanide can be expected when a Ringer's bicarbonate solution is employed with carbon dioxide in the gas volume. To support this he cites the work of Alt (1930) who with this technique showed complete inhibition of the oxygen uptake of kidney, liver and spleen tissue in the presence of 1/100 N KCN.

It was thought that if phosphate does injure the cells so as to affect the influence of cyanide on the amount of oxygen taken up, this could easily be shown by varying the phosphate concentration in the Ringer's solution employed. Consequently the oxygen uptake of rat liver tissue inhibited by M/150 cyanide was compared employing normal Ringer's solution, Ringer's solution with no phosphate, Ringer's solution containing a M/15 phosphate concentration and Ringer's solution containing 0.02 M bicarbonate instead of phosphate. It was found that the oxygen uptake inhibited by the cyanide was not such as to indicate that changing the phosphate concentration has any influence. That is, the modified Ringer's solution containing no phosphate and the modified Ringer's solution containing 0.02 M $NaHCO_3$ instead of phosphate gave over a two hour period 38 and 58 per cent inhibition of the oxygen uptake respectively, as compared to 56 per cent with a M/15 phosphate concentration. It should be pointed out that the modified Ringer's solution containing the 0.02 M $NaHCO_3$ was not equilibrated with a 40 mm. CO_2 tension and consequently the pH was elevated somewhat. In this connection it would be highly desirable to have data employing the Warburg (1924) technique with CO_2 in the gas phase over the bicarbonate Ringer's solution. Some effort was made to duplicate this technique. The data must be omitted since they were inconsistent and inconclusive. That is, employing the vessels at hand, irregular pressure changes were obtained in the vessel containing the larger fluid volume. This was undoubtedly due to incomplete equilibration of the fluid with the CO_2 oxygen mixture prior to making the first readings. At the present time vessels of the same size and description as employed by Warburg are being prepared and observations employing such vessels will appear in a forthcoming paper. It should be pointed out that the results of Banga, Schneider and Szent Györgyi (1931a) agree with those of Dixon and Elliott. These authors also found that varying the medium in which the tissue was suspended had no definite influence on the cyanide sensitivity.

O_2 uptake of rat tissue in the presence of urethane, pyrophosphate and arsenite. In order to determine whether various rat tissue behaved differently when suspended in Ringer's solution containing substances known to affect the oxygen uptake, observations were made employing 5 per cent urethane, M/15 sodium pyrophosphate and arsenite. To conserve space in the JOURNAL, the tabular data will be omitted. Urethane in 5 per cent concentrations inhibited the oxygen uptake of rat liver, kidney, spleen and muscle on the average from 63 to 83 per cent. Further, urethane plus M/150 cyanide had essentially the same effect on the oxygen uptake of these tissues as urethane alone. (The percentage inhibition range was 63 to 81 per cent.)

Somewhat similar results were obtained with M/15 sodium pyrophos-

phate. The per cent inhibition of the oxygen uptake by M/15 sodium pyrophosphate for the same rat tissues varied from 48 to 68 per cent, while with pyrophosphate plus M/150 cyanide the values varied from 48 to 76 per cent.

Arsenite in 0.005 M concentration inhibited the oxygen uptake of rat liver, spleen and kidney over a 90 minute period from 75 to 88 per cent. For the same tissues and the same time interval, arsenite in 0.001 M concentration gave an inhibition from 62 to 70 per cent.

O_2 uptake of rat tissue in the presence of paraphenylenediamine. Banga, Schneider and Szent Györgyi (1931a) studied the effect of cyanide on the oxygen uptake of tissue in the presence of paraphenylenediamine. A number of trials were made employing concentrations of cyanide from M/1000 to M/100 in the presence of the diamine. In all cases the oxygen uptake of the system muscle plus the diamine and cyanide was brought back to the value of the muscle alone with cyanide and without the diamine. This they point out agrees with the results of Warburg and Keilin and conclude that if the action of HCN on the O_2 activation alone is desired then the concentration of HCN cannot be greater than M/1000 since with higher concentrations the H activation is also inhibited.

A number of experiments were performed to observe the influence of cyanide on the oxygen uptake of fresh rat liver, kidney and spleen tissue and tissue heated one and one-half hours at 52°C. when in the presence and absence of paraphenylenediamine. The data obtained are presented in table 2. It is observed that the heating of tissue at 52°C. for one and one-half hours markedly decreased the oxygen uptake. Further, cyanide did not eliminate this residual oxygen uptake. As a matter of fact in half of the observations the oxygen uptake of the heated tissue in the presence of cyanide had a slightly greater value than in the absence of the cyanide. Employing spleen and kidney the oxygen uptake of the fresh tissue in the presence of the diamine was slightly less than was found with the tissue alone. Fresh liver tissue in the presence of the diamine had a greater oxygen uptake than without. Even though the oxygen uptake of the heated tissue was markedly decreased, in the presence of the diamine the oxygen uptake was increased in every instance. In the presence of the diamine and cyanide, all three of the tissues studied (whether heated or not), showed a decrease in the oxygen uptake when compared to the tissue plus diamine alone. Liver tissue on the whole showed the same oxygen uptake when in the presence of 0.001 M cyanide and diamine as with the cyanide alone. Cyanide in 0.001 M concentration had a marked influence on the oxygen uptake of heated kidney tissue in the presence of the diamine, while the influence was not so pronounced with the fresh tissue. Spleen tissue plus the diamine and 0.001 M cyanide had less oxygen uptake than with the diamine alone, however, it appeared that a higher concentration

of cyanide (0.005 M) was necessary to completely inhibit the oxygen uptake caused by the presence of the diamine and to bring it back to the value obtained with the tissue and cyanide alone.

It should be pointed out that all the values obtained for the oxygen uptake of tissues in the presence of the diamine had to be corrected by a blank. The diamine employed had an oxygen uptake itself and consequently a blank was determined employing the same solutions minus the tissue. The data obtained above, however, on the whole agree with those reported

TABLE 2

O_2 uptake of rat tissue in the presence of para-phenylenediamine and cyanide

TISSUE	CONCENTRATION OF CYANIDE	O_2 UPTAKE OVER A 60 MINUTE INTERVAL				REMARKS
		Normal Ringer's	Ringer's plus cyanide	Ringer's plus diamine	Ringer's plus cyanide and diamine	
	M.	*cu. mm./mgm. dry tissue*				
Spleen	0.001	9.54	3.18	9.23	3.92	Fresh tissue
Spleen	0.001	1.46	2.13	7.24	5.40	Tissue heated*
Spleen	0.002	10.10	2.41	9.68	5.48	Fresh tissue
Spleen	0.002	0.97	1.49	6.25	2.76	Tissue heated
Spleen	0.005	8.76	3.15	6.10	3.29	Fresh tissue
Spleen	0.005	0.41	0.31	9.34	1.10	Tissue heated
Liver	0.001	9.71	4.97	15.50	2.94	Fresh tissue
Liver	0.001	0.81	0.94	4.78	2.16	Tissue heated
Liver	0.002	6.88	2.29	13.05	2.36	Fresh tissue
Liver	0.002	2.03	1.51	6.74	2.29	Tissue heated
Liver	0.005	5.73	1.80	7.91	1.60	Fresh tissue
Liver	0.005	0.61	0.44	3.39	0.33	Tissue heated
Kidney	0.001	12.15	7.05	11.32	10.32	Fresh tissue
Kidney	0.001	0.73	1.55	8.14	2.88	Tissue heated
Kidney	0.002	12.45	4.07	12.15	4.35	Fresh tissue
Kidney	0.002	0.33	0.45	10.20	0.20	Tissue heated
Kidney	0.005	15.60	3.96	11.66	2.00	Fresh tissue
Kidney	0.005	1.18	0.96	7.68	1.61	Heated tissue

* Tissue was heated for one and one-half hours at 52°C.

by Banga, Schneider and Szent Györgyi (1931a), namely, that oxygen activation as measured by the ability to oxidize paraphenylenediamine is inhibited by small concentrations of cyanide.

The ability of rat liver, kidney and spleen tissue to oxidize the diamine was studied further when either urethane, pyrophosphate or arsenite was present in the system in addition to the diamine. From the data in table 3 it is observed that urethane in 5 per cent concentration seemed to increase the amount of the oxygen uptake of spleen tissue plus the diamine. With

liver and kidney the oxygen uptake with urethane and diamine was somewhat less than with tissue and diamine alone, but much greater than with tissue and urethane. All three of the rat tissues studied when in the presence of diamine and M/15 sodium pyrophosphate showed a decrease in the oxygen uptake when compared to that found with the tissue and diamine alone. In the case of spleen and kidney tissue the system tissue plus diamine and pyrophosphate showed slightly less oxygen uptake than the tissue and pyrophosphate alone. With liver tissue the system tissue plus diamine and pyrophosphate took up slightly more oxygen than the tissue and pyrophosphate alone. Arsenite in 0.002 M concentration,

TABLE 3

O_2 uptake of rat tissue in the presence of paraphenylenediamine and in addition either urethane, sodium pyrophosphate or arsenite

TISSUE	O_2 UPTAKE OVER A PERIOD OF 60 MINUTES				REMARKS
	Normal Ringer's	Ringer's plus substance	Ringer's plus diamine	Ringer's, substance and diamine	
	cu. mm./mgm. dry tissue				
Spleen	8.10	3.53	5.71	9.50	Substance, urethane 5 per cent
Kidney	12.10	1.69	12.77	9.61	Substance, urethane 5 per cent
Liver	6.44	1.65	9.08	5.50	Substance, urethane 5 per cent
Spleen	9.63	3.88	9.60	2.58	Substance, M/15 sodium pyrophosphate
Kidney	9.85	4.23	7.98	2.30	Substance, M/15 sodium pyrophosphate
Liver	5.90	3.06	14.37	5.47	Substance, M/15 sodium pyrophosphate
Spleen	5.27	1.55	8.89	5.82	Substance, 0.002 M arsenite
Kidney	9.27	3.02	18.20	18.20	Substance, 0.002 M arsenite
Liver	5.72	2.61	6.39	12.90	Substance, 0.002 M aresnite

even though markedly decreasing the oxygen uptake of the tissue, had little effect on the oxygen uptake of the tissue when in the presence of the diamine.

Discussion. It would seem that the data presented in this report agree with the conclusions of Dixon and Elliott (1929) that the respiration (rate of oxygen uptake) of rat tissues, at least when the tissues are suspended in phosphate Ringer's or in bicarbonate Ringer's not under a 40 mm. CO_2 tension, is made up of two parts. One part is inhibited by cyanide while the other part is cyanide stable.

In this connection the observations and conclusions of Kuhn and Meyer (1929) are of interest. These workers observed the oxidation of various

substrates in the presence of pyridine-hematin and suggested that the cyanide sensitivity is largely dependent upon the chemical nature of the substrate. They suggest in explaining the observations of Dixon and Elliott that cyanide might inhibit the oxidation of carbohydrates and yet have little or no influence on the oxidation of fats. In support of this they further point out that Dixon and Elliott observed a simultaneous lowering of the respiratory quotient.

From the observations of Banga, Schneider and Szent Györgyi (1931a) probably no correlation between the degree of inhibition of the oxygen uptake employing M/150 cyanide and the extent of indophenol blue produced by the "Nadi" reagent can be expected. According to the above workers, and as observed in the present study, the indophenol oxidase is inhibited by much smaller amounts of cyanide. In addition, the above workers point out that with higher concentrations the hydrogen activation is also inhibited. Further, the amount of reducing substances present in the tissue may affect the amount of indophenol blue produced (Keilin, 1929). In spite of this, however, some correlation might have been found had smaller concentrations of cyanide been employed, say 0.001 M. It is of interest in this connection that Dixon and Elliott (1929) found that spleen tissue was less sensitive to smaller concentrations of cyanide than the other tissues studied, and, as has been our observation, this tissue gives comparatively little color employing the "Nadi" reagent.

Keilin (1929) studying the indophenol oxidase activity in heart muscle preparations found that M/1000 KCN inhibits completely the indophenol reaction as well as the oxygen uptake of muscle preparations in the presence of para-phenylenediamine. Further, ethyl urethane in 5 per cent concentration and N/30 sodium pyrophosphate had no effect on the activity of the oxidase. The oxygen uptake was inhibited by 18 per cent when N/15 sodium pyrophosphate was employed. In the present study urethane in 5 per cent concentration, although inhibiting the oxygen uptake of the rat tissues studied, had comparatively little influence on the oxygen uptake of the system tissue plus urethane and paraphenylenediamine. The oxygen uptake of the system tissue plus diamine and M/15 sodium pyrophosphate was definitely inhibited when compared with the system tissue plus diamine. This would lead to the conclusion that sodium pyrophosphate (M/15) inhibits the indophenol oxidase system.

It has been shown by Szent Györgyi and his co-workers (1931b) that by employing small concentrations of arsenious acid the respiration of tissues can be sharply divided into two parts, and further, that the arsenious acid has no influence on oxygen activation but that its action is probably on hydrogen activation. The results obtained in this report agree with this conclusion insofar that it has been found that arsenite in small concentrations markedly inhibits the oxygen uptake of rat liver, kidney

and spleen tissue and yet does not influence the ability of these tissues to take up oxygen in the presence of paraphenylenediamine.

SUMMARY

The effect of M/150 cyanide on the oxygen uptake of rat tissue suspended in phosphate Ringer's solution was compared with the extent of indophenol blue produced by employing the "Nadi" reagent. The oxygen uptake of the tissue and the degree of inhibition of oxygen uptake by cyanide bore no relation to the amount of indophenol blue produced.

The data obtained on the influence of cyanide on the oxygen uptake of rat tissue agree with the conclusion of Dixon and Elliott that cyanide does not completely inhibit the oxygen uptake of rat tissue slices when suspended in phosphate Ringer's solution.

Urethane in 5 per cent concentration inhibited the oxygen uptake of rat liver, kidney, spleen and muscle on the average from 63 to 83 per cent. Cyanide (M/150) plus urethane gave essentially the same inhibition.

Sodium pyrophosphate (M/15) on the same rat tissues inhibited the oxygen uptake from 48 to 68 per cent. Cyanide plus sodium pyrophosphate gave from 48 to 76 per cent inhibition.

Heating rat liver, kidney and spleen tissue at 52°C. for one and one-half hours, although markedly decreasing the oxygen uptake of the tissue, did not destroy the ability of the tissue to take up additional oxygen in the presence of paraphenylenediamine.

Cyanide in small concentration inhibited the ability of rat liver, kidney and spleen tissue to take up additional oxygen in the presence of paraphenylenediamine. Spleen tissue appeared to take slightly higher concentrations of cyanide for this inhibition than the other tissues.

Urethane in 5 per cent concentration did not inhibit the ability of these three rat tissues to take up oxygen in the presence of paraphenylenediamine.

Sodium pyrophosphate (M/15) inhibited the ability of these three rat tissues to take up oxygen in the presence of paraphenylenediamine.

Arsenite (0.002 M) although markedly decreasing the oxygen uptake of rat liver, kidney and spleen tissue had no influence on the ability of these tissues to take up oxygen in the presence of paraphenylenediamine.

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THE EFFECT OF INORGANIC IRON WITH AND WITHOUT ULTRAVIOLET IRRADIATION UPON THE PREVENTION AND CURE OF NUTRITIONAL ANEMIA

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The effect of radiant energy upon Fe metabolism in nutritional anemia of the rat seems to have been little studied. Foster (1) produced anemia in rats by milk feeding and observed the effect of radiant energy in preventing or curing this type of anemia, using the "Pan-Ray" carbon arc and the air-cooled Cooper-Hewitt quartz mercury vapor lamp. This treatment had a slight but definite effect in increasing the Hb, number, size and saturation of the red cells. The effects of radiant energy upon the blood has recently been summarized very completely by Laurens (2).

In our previous work in nutritional anemia of the rat in Cleveland three anemic rats were fed upon milk alone, without any inorganic supplements, and were given ¾ hour irradiation daily with the General Electric Sunlight Mazda Lamp at a distance of 4 feet. To our surprise one of these rats increased in red cells from 1.77 to 10.30 million per c.mm. and from 3.6 to 12.5 grams Hb per 100 cc. in 12 weeks. Another increased in red cells (microcytes) from 2.36 to 11.45 million per c.mm. in one week and at the end of 12 weeks the red cell count was 8.24 million per c.mm. with no increase in Hb. The third animal showed no increase in either cells or Hb.

This unusual response in red cells in our rats under the influence of radiant energy led us to make a detailed study of the effect of this type of energy upon Fe metabolism in the prevention and cure of nutritional anemia in the rat, the results of which are given below.

EXPERIMENTAL. The experimental procedures were the same as previously described by Beard and Myers (3). All animals were kept in new galvanized iron cages (which were covered with aluminum paint) on the desks in the large laboratory. Daily doses of Fe (H_2S filtrate of electrolytic Fe) from 0.05 to 0.30 mgm. were fed to the different groups of control and experimental animals and the weekly response in Hb and cells noted. The experimental animals were moved in their cages to a large hood at the side of the laboratory and given ¾ hour irradiation daily from the General Electric Sunlight Mazda Lamp, type S-I, at a distance

of 4 feet. This lamp has recently been described by Carter (4). In the preventive experiments normal young rats at weaning were given the same treatment received by the animals above.

The Cu content of our Fe solution and milk was determined by Dr. Fred Allison using his magneto-optic method (5).[1] The solids from a liter of milk contained 6.9×10^{-8} grams of Cu; the Fe solution, 4×10^{-6} grams per cc. With a daily milk intake of 50 cc. with 0.25 mgm. Fe the rats would be receiving 0.0004184 mgm. of Cu. A water solution containing 0.01025 mgm. Cu per cc. was found by Allison to contain 0.01200 mgm. per cc. The low Cu content of the milk might be due to the result of a volatilization of some of the Cu in the ashing process but this seems hardly probable, since this same method of ashing the milk solids is usually done in the procedure for the colorimetric analysis of Cu in milk.

RESULTS. The average results obtained in the curative studies are given in table 1.[2] With milk alone the level of Hb remained about the same, with a slight increase in red cells. With milk and irradiation many of the animals died and the decrease in Hb and cells was greater in this group than in the group with milk alone. The deaths here were likely due to the fact that these animals refused milk at the beginning of the experiment, since no deaths occurred in other animals with a very few exceptions, where the milk intake was adequate.

With milk and 0.05 mgm. Fe daily there was a slight increase in cells, but most of the animals died. There was a noticeable effect of irradiation on both the increase in cells and Hb, and death was prevented in all animals of this group.

In all the remaining groups (0.10 to 0.30 mgm. Fe with irradiation) a slightly greater Hb recovery took place than occurred on Fe alone in the 0.20 and 0.25 mgm. groups. There was, however, a definite increase in regeneration of the red cells, in all of these groups with the exception of the 0.10 and 0.25 mgm. doses, which was due to the irradiation. It is well to bear in mind, however, that the animals during irradiation were generally much more active than their non-irradiated controls. There was also a direct relation between the Fe intake and the increase in body weight.

The daily milk intake was observed with 51 animals reported in this

[1] See also note by Bishop, Otto and Baisden (J. Amer. Chem. Soc., **56**, 408, 1934) in this connection.

[2] In our preliminary publication of these results (Proc. Soc. Exp. Biol. and Med., 1933, **31**, 26) only the beginning and highest values of cells and Hb, with the time necessary to reach same, were given. A comparison of these highest values obtained with Fe, with and without irradiation, forms the basis for the conclusions given in the present paper. It has also seemed of importance to determine if these beneficial effects were permanent. We give, therefore, in table 1 the values that were present at the end of the 10-week experimental period. This makes the results of all experimental groups comparable since they were all run for the same length of time.

TABLE 1

Effect of inorganic iron and irradiation in the cure of nutritional anemia. Average values

NUMBER OF RATS	AVERAGE DAILY MILK	BODY WEIGHT		HB per 100 cc.			R.B.C.			HB		R.B.C.		DIET
		Beginning	End	Beginning	Highest value	End	Beginning	Highest value	End	Highest value	End	Highest value	End	
	cc.	*grams*	*grams*	*grams*	*grams*	*grams*	*m. per c.mm.*	*m. per c.mm.*	*m. per c.mm.*	*wks.*	*wks.*	*wks.*	*wks.*	
10	38	92 0	160 5	3 9	3 7	3 6	3.55	4 80	4 38	8 6	8 6	6 8	8 6**	Milk alone
10	22	97 0	93 9	4 0	4 3	3 6	3 11	3 67	2 75	3 6	6 7	2 7	6 1**	Milk plus ¾ hr. irradiation daily
5	33	66 4	107 5	3 0	3 2	3 2	2 49	3 30	2 76	5 0	5.0	3 0	5 0**	Milk plus 0.05 mgm. Fe daily
9	39	88 5	146 4	3 5	5 6	4 3	3 01	5.10	4 90	7 3	10 0	7 0	10 0	Milk plus 0.05 mgm. Fe plus ¾ hr. irradiation daily
3	38	67 0	181 3	3 0	7 8	7 4	2 41	8 99	8.22	9.6	10 0	8 0	10 0	Milk plus 0 1 mgm. Fe daily
7	38	99.0	192 1	3 5	8 6	7 4	2 62	7 75	7.42	5.6	10.0	8 9	10 0	Milk plus 0 1 mgm. plus ¾ hr. irradiation daily
6	55	69 9	205 7	3 0	7 2	6 6	2 81	8 23	6 99	8 0	10 0	8 3	10 0	Milk plus 0.15 mgm. Fe daily
8	54	92.4	169 1	4.6	8 9	7 5	2 95	9 46	9.24	6 5	10.0	9 4	10.0	Milk plus 0.15 mgm. Fe plus ¾ hr. irradiation daily
6	50	68 6	192 5	3 0	9 9	9 6	2.57	9 95	9.94	9 3	10 0	9 9	10 0	Milk plus 0.20 mgm. Fe daily
6	49	95.5	170 0	3.7	12.9	10 2	2 58	11 48	10 90	8 0	10 0	7 7	10 0	Milk plus 0.20 mgm. Fe plus ¾ hr. irradiation daily
4	51	64 6	178 7	4 2	9 7	8 4	2 61	9 72	8.59	7 5	10 0	9 0	10 0	Milk plus 0.25 mgm. Fe daily
9		81.4	155.1	3.4	11.1		2.71	10.40		6 6		6 9		Milk plus 0.25 mgm. plus ¾ hr. irradiation daily*
5	45	57.1	186 8	3 0	10 5	9 8	3 01	10 20	9 17	8 6	10 0	8 2	10 0	Milk plus 0.3 mgm. Fe daily
10	54	74 9	204.2	3 1	10 0	9 7	2 74	10 37	9 92	7 5	10 0	8 6	10 0	Milk plus 0.3 mgm. Fe plus ¾ hr. irradiation daily

* The animals in this group happened to be run for different lengths of time, hence the "highest values" are comparable but the "end" values are not.

** These are the average survival periods of the animals in these groups.

paper. There was a direct relation between the daily dose of Fe ingested, (from 0 to 0.15 mgm.) to the daily milk intake (33 to 55 cc.). Irradiation did not increase the milk intake over that obtained with Fe alone. The milk intake in all other experiments had no influence upon the results obtained.

TABLE 2

Effect of inorganic iron and irradiation in the prevention of nutritional anemia. Average values

NUMBER OF RATS	AVERAGE DAILY MILK	BODY WEIGHT		HB PER 100 CC.		R.B.C. PER C.MM.		DURATION OF EXPERIMENT		DIET
		Before	After	Before	After	Before	After	Hb	R.B.C.	
	cc.	*grams*	*grams*	*grams*	*grams*	*millions*	*millions*	*weeks*	*weeks*	
6		48.0	102.0	10.6	3.9	7.46	3.68	5.0	5.0	Milk alone
13		50.0	105.0	9.1	4.2	6.54	3.97	5.0	5.0	Milk plus ¾ hr. irradiation daily
9		49.0	107.0	8.9	4.3	7.10	4.30	5.0	5.0	Irradiated milk alone
5	26	28.3	82.0	5.9	3.0	5.05	2.96	7.6	7.6	Milk plus 0.05 mgm. Fe daily*
11	32	31.4	122.7	8.0	6.5	4.63	6.15	10.0	10.0	Milk plus 0.05 mgm. Fe plus ¾ hr. irradiation daily
4	36	32.8	107.4	5.4	5.4	5.64	5.25	10.0	10.0	Milk plus 0.1 mgm. Fe daily
9	37	32.2	127.1	7.4	6.8	4.83	7.23	10.0	10.0	Milk plus 0.1 mgm. Fe plus ¾ hr. irradiation daily
5	53	40.8	185.2	9.6	10.4	5.47	8.87	10.0	10.0	Milk plus 0.15 mgm. Fe daily
10	47	32.5	169.0	7.8	9.0	4.53	8.70	10.0	10.0	Milk plus 0.15 mgm. Fe plus ¾ hr. irradiation daily
5	47	29.0	176.8	9.3	11.0	5.33	8.06	10.0	10.0	Milk plus 0.20 mgm. Fe daily
10	47	36.8	167.4	9.0	11.1	5.25	9.31	10.0	10.0	Milk plus 0.20 mgm. Fe plus ¾ hr. irradiation daily
5	56	35.5	213.4	8.8	12.7	5.27	9.33	10.0	10.0	Milk plus 0.25 mgm. Fe daily
10	48	33.0	182.3	9.0	12.1	5.37	8.62	10.0	10.0	Milk plus 0.25 mgm. Fe plus ¾ hr. irradiation daily
5	39	32.8	134.7	6.8	8.0	5.00	7.49	10.0	10.0	Milk plus 0.3 mgm. Fe daily
10	51	36.1	155.6	8.0	10.1	4.71	8.52	10.0	10.0	Milk plus 0.3 mgm. Fe plus ¾ hr. irradiation daily
10	47	27.7	166.8	5.7	11.1	4.61	9.67	10.0	10.0	Irradiated milk plus 0.3 mgm. Fe daily

* Three rats in this group died.

The results obtained in the preventive studies are given in table 2. With milk alone there was a marked drop in both cells and Hb. When the animals were irradiated the drop in Hb was about the same, but there was a slight effect upon the drop in cells (−50.6 per cent as compared with

−39.2 per cent). Irradiated milk gave exactly the same results as milk plus irradiation of the rat.

With 0.05 mgm. Fe alone there was a drop of 49 per cent in Hb and 41 per cent in red cells. This dose with irradiation resulted in a drop of only 18 per cent in Hb and an increase of 33 per cent in red cells. This effect on the cells was striking. Five animals of the latter group also showed good Hb values.

With daily doses of Fe from 0.10 to 0.30 mgm. irradiation had no beneficial effect upon the increase in Hb over that obtained with these doses of Fe alone. Irradiation, however, with daily doses of Fe from 0.10 to 0.30 mgm. gave increases from 23 to 62 per cent in red cells, with one exception, over that obtained with Fe alone.

The effect of irradiated milk was even more striking on the increase in cells and Hb than was irradiation of the rat, 5 cases of polycythemia occurring in this group. There was a 77 per cent increase in Hb and 60 per cent increase in cells in favor of the irradiated milk group, as compared to the control group receiving 0.30 mgm. Fe alone.

Discussion. It will be observed from the results given in table 1 that we are obtaining a somewhat slower response upon pure Fe alone than that obtained in previous work (3). We believe that this was due to a lack of vitamin D in the milk used and an average daily milk intake (35–50 cc.) below the optimum (50–75 cc.) necessary for Hb recovery in 6 weeks. It will also be noticed from the table that the animals receiving Fe and irradiation were heavier at the beginning of the experiment than those controls receiving Fe alone. This was due to the fact that the former animals usually required a longer time to become anemic than the others, thus allowing more time for growth before the experiments were begun. For this reason in our present studies we may not have animals of comparable body weights at this time. Differences in body weight cannot, however, account for the differences in red cell response observed in this study. The experimental periods were begun when the red blood cell count was about 3 million per c.mm. and Hb below 4 grams per 100 cc. regardless of the body weight of the animal.

According to Laurens (2) contradictory evidence of the effects of ultraviolet energy upon the red cell count has been observed. Eleven investigators noted no change in the erythrocyte count due to irradiation while 18 reported an increase. These conflicting results were, no doubt, due to the fact that no two investigators used the same source of energy in the same way and the intensity of the irradiation was usually not measured. Conflicting results are, therefore, to be expected.

Recent work by Eveleth, Bing and Myers (6) and Keil and Nelson (7) shows that injected Fe will cause Hb regeneration and that the action of Cu is probably to assist in the absorption of Fe. After this manuscript

was written Myers and co-workers expressed the view, based upon the work of Foster (1), that the beneficial effect of irradiation in nutritional anemia of the rat might be due to its effect in facilitating the absorption and utilization of Fe similar to the action of vitamin D in Ca and P metabolism. The results of our work suggest that this may be the case. It is practically certain that the beneficial effects of irradiation noted were due to the formation of vitamin D in the animals and milk. However, since the exact function of this vitamin in Ca and P metabolism is still largely unknown, it would seem premature at this time to subscribe to this theory.

Irradiation has also been employed in other anemic states and in other species of animals (2). Laurens and co-workers (8) (9) studied the influence of irradiation upon hemorrhagic and hemolytic anemia in the dog. In hemorrhagic anemia the most pronounced effect was observed upon the red cells and reticulocytes, with no effect upon Hb. In hemolytic anemia an increase in pigment was also observed. Hobert (10) irradiated normal mice for 2 minutes with a quartz-mercury lamp. There was a ten fold increase in the erythrocytes. In mice made anemic by bleeding irradiation up to 16 minutes caused an increase in both cells and Hb. If a few days were allowed between irradiations the cells and Hb rose even above their normal value. Mathews, Doyle, and Whiting (11) observed a greater incidence of anemia in pigs reared in a central hog house as compared to those reared out of doors.

Osato and Tanaka (12) studied the effect of ultraviolet irradiation on blood regeneration and Fe metabolism in dogs, guinea pigs and rats. They concluded that blood Fe increases at the expense of liver and marrow Fe. The feeding of a product rich in Fe resulted in a deposit of this element in the spleen and liver which disappeared after irradiation along with marked blood regeneration. They also believe that irradiation withdraws Fe from different organs for the formation of Hb. van Creveld and Heybroek (13) observed that a combination of liver and Fe, or of reduced Fe and vitamin D, prevented the onset of severe grades of anemia in premature infants.

A few of our experimental groups of animals on Fe and irradiation did not regenerate Hb and cells as rapidly as those on the dose of Fe alone. But when the average results of all groups of experimental animals are compared to those of their controls it is clearly seen that irradiation with Fe was much more beneficial in red cell production in the prevention and cure of nutritional anemia than was the dose of Fe alone. This does not mean that irradiation is necessary with Fe, but simply enhances its action in this connection. In both preventive and curative studies the effect of irradiation upon Hb increase became, in general, less apparent due to the effect of the increasing doses of Fe given.

SUMMARY

The effect of inorganic Fe, with and without 45 minutes' ultraviolet irradiation daily from the General Electric Sunlight Mazda Lamp, type S-I at a distance of 4 feet upon blood formation in the prevention and cure of nutritional anemia in the rat has been studied. The results obtained were as follows:

Curative studies. 1. The effect of ultraviolet irradiation in the curative studies was most marked with 0.05 mgm. Fe daily. Substantial increases in Hb and red cells were noted over that obtained upon this dose of Fe alone. Death, which usually occurred on this small dose of Fe, was prevented by the addition of the radiant energy.

2. Pure Fe gave an increase in Hb in direct relation to the dose fed from 0.1 to 0.3 mgm. daily, with one exception (0.15 mgm.). The difference in Hb response when 0.05 and 0.10 mgm. Fe were fed, representing an average increase from 7.0 to 160.0 per cent, was striking. The anemic young rats also recovered a normal cell count on daily doses of Fe from 0.10 to 0.30 mgm.

3. A polycythemia was observed in 10 animals on Fe alone and in 22 on Fe and irradiation. In a few cases it was temporary, but in most of the others it was still present at the end of the 10 weeks' experimental period.

4. Irradiation had no influence on Hb recovery better than that obtained on Fe alone, from 0.1 to 0.3 mgm., with two exceptions (the 0.20 and 0.25 mgm. doses). There was a definite beneficial effect upon red cell production with Fe and irradiation as compared to Fe alone, with the exception of the 0.10 and 0.25 mgm. doses. The outstanding effect of irradiation of the rat was upon the red cell rather than upon Hb regeneration.

5. A close relationship between the increase or decrease in body weight and Hb regeneration was noted.

Preventive studies. 1. Hb on 0.05 mgm. Fe daily fell about 49 per cent; with this dose of Fe and irradiation the drop was 18 per cent. The red cells decreased 41 per cent on this dose of Fe alone and increased 33 per cent when irradiation also was given the animals.

2. Doses of Fe alone from 0.15 to 0.3 mgm. daily prevented the onset of anemia.

3. Irradiation with 0.1 mgm. Fe resulted in a 50 per cent increase in red cells compared to that obtained with this dose of Fe alone. With 0.15 to 0.30 mgm. Fe and irradiation there was no definite effect on the increase in Hb but a substantial effect upon the increase in erythrocytes, with one exception.

4. The effect of ingestion of irradiated milk with 0.3 mgm. Fe daily was more striking than irradiation of the rat, resulting in a 77 per cent increase in Hb and a 60 per cent increase in red cells, over that obtained with this

amount of Fe alone. Several cases of polycythemia also occurred in these studies.

We wish to express our thanks to the American Academy of Arts and Sciences for a grant from their Permanent Science Fund for these studies. We also wish to express our thanks to Dr. Fred Allison of the Alabama Polytechnic Institute for his determinations of Cu in our Fe solutions and milk by his magneto-optic method.

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THE CREATININE CLEARANCE AS A MEASURE OF GLOMERULAR FILTRATION IN DOGS WITH PARTICULAR REFERENCE TO THE EFFECT OF DIURETIC DRUGS

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Since the appearance of Rehberg's work (1926) on the use of the creatinine clearance as a measure of glomerular filtration, a number of studies of kidney function using this procedure have been reported. The method is based on the premise that creatinine is excreted solely by filtration through the glomeruli of the kidney and undergoes no reabsorption in the tubules. The total amount of urinary creatinine is estimated and the glomerular filtration necessary for its elaboration is calculated from the formula

$$F = \frac{C\,u}{C\,b} \cdot V$$

where F represents the glomerular filtration, Cu the concentration of creatinine in the urine, Cb the concentration in the blood, and V the volume of the urine in cubic centimeters per minute.

The original premises of Rehberg have been supported by Ekehorn (1931), who argues from *a priori* grounds that creatinine is a no-threshold substance; but this author has offered no experimental proof as to the mode of excretion of creatinine. Perhaps the best indirect evidence in favor of the use of the creatinine clearance as a measure of filtration, is that of Poulsson (1930) who has found similar values for the clearance of glucose and creatinine in phlorhizinized dogs. His methods and conclusions have been adversely criticized by Shannon, Jolliffe, and Smith (1932) who have found that the clearance values for xylose and glucose in dogs poisoned by phlorhizin are identical, and that the creatinine clearance before phlorhizin is given averages forty per cent higher than the xylose clearance.

Rehberg (1926) working on himself, and Fremont-Smith and others (1930) working on normal subjects and patients with edema, have reported no increase in the amount of glomerular filtrate[1] with water diu-

[1] In this paper the term "glomerular filtrate" is used synonymously with creatinine clearance when such a relation is implied by the authors who are quoted.

resis. Lassen (1932b) working with normal human subjects has found decreases in filtration with diuresis following salyrgan.

In regard to the effect of diuretic drugs on the creatinine clearance, Chrometzka and Unger (1931) have found a constant fall in this clearance in humans during the course of diuresis following salyrgan, novasurol, diuretin, urea, and thyroid as well as during a control study. Lassen (1932a) has reported that in patients with edema of cardiac origin, who show low initial filtration values, there is a decided increase in the filtration with salyrgan diuresis, amounting to 319 per cent in one case and averaging 46 per cent in his series. On the other hand, Schmitz (1932) working with normal dogs and Herrmann and others (1932) working with edematous patients have reported no increase in filtration after salyrgan, but a marked increase with euphyllin diuresis. Blumgart and others found no change in filtration in normal men with diuresis from euphyllin, salyrgan or novasurol.

This present study was carried out with the purpose of determining the effect of diuresis following drugs on the creatinine clearance in dogs. Additional observations made during the course of these experiments are reported elsewhere (Fulton et al.).

PROCEDURE. The experiments were performed upon seven female dogs weighing between 12 and 17 kgm. Food was withheld on the mornings of the experiment. At the beginning of the test 6.6 cc. of water per kilogram of body weight were given by stomach tube and like amounts were given each hour for a total of five hours. Two hours after the beginning of the experiment, a diuretic agent was given. Blood and urine samples were taken each half-hour after the first hour, for a total of five hours. The blood was withdrawn from the leg veins by venepuncture and oxalate used to prevent coagulation. Urine was obtained by catheter with gentle pressure over the abdomen to secure complete emptying. Calculations of the creatinine clearance were made from simultaneous analyses of the blood and urine. The blood concentration values were interpolated to represent the middle of each half-hour period.

The creatinine determinations were carried out after the method of Folin and Wu (1919) as modified by Harper and Butler who substituted a solution of sodium picrate for the picric acid of the original procedure. The actual procedure as used by us follows:

To 5.0 cc. of 1:10 tungstate filtrate of laked whole blood 2.0 cc. of a freshly prepared solution of 1.6 per cent sodium picrate containing one-third volume of 10 per cent sodium hydroxide, were added. Standard solutions of creatinine were treated in a similar manner and readings made in the Duboscq colorimeter after ten minutes. Urine samples estimated to contain 1 to 2 mgm. of creatinine were transferred to 100 cc. volumetric flasks. To these were added 20.0 cc. of a solution of 1 per cent sodium picrate, containing one-tenth volume of sodium hydroxide. Dilutions to volume were made after ten minutes and color comparisons with similarly treated standard solutions were carried out in the usual manner.

The earlier results were obtained with the normal blood levels of creatinine, employing correction curves for the color due to the alkaline picrate as suggested by Hunter and Campbell (1917). However, because of the work of Behre and Benedict (1922) who have questioned the existence of creatinine in normal blood, and of Gaebler and Keltch (1928) and Gaebler (1930) who have demonstrated the lack of specificity of the Jaffe reaction, the later results were determined after giving 2 to 3 grams of creatinine by mouth at the beginning of the experiment. This produced higher levels of concentration of creatinine in the blood and the readings were more accurate. (Even at these concentrations the recovery of known amounts of creatinine added to the blood carries a range of error of 5 per cent.) In the beginning the readings were made on plasma but the excessive amounts of blood necessary for multiple determinations led to the use of whole blood values.

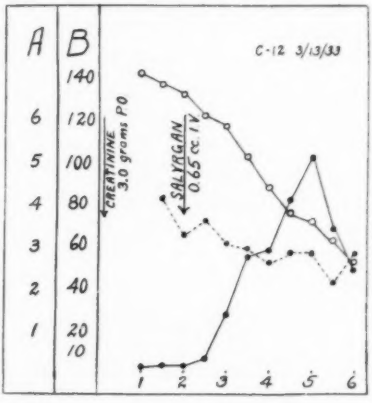

Fig. 1. Abscissa: Time in hours. Ordinate: A. Urine volume in cc./min. ●——●——●
B. { Blood creatinine in mgm./liter ○——○——○ Creatinine clearance in cc./min. ●----●----●

The results of a typical experiment are shown in figure 1. The change in the urine volume as a result of the diuresis, from 0.29 cc. per minute to 5.06, an increase of nearly twenty-fold, was not reflected by a rise in the creatinine clearance, the latter actually decreasing with the fall in blood concentration, even in the face of the marked rise in urine volume. Similar results were obtained in all of the experiments in which different diuretics were used. These observations were repeated on seven dogs, using theophylline-ethylene-diamine eighteen times, salyrgan fifteen, novasurol eight, theocin six, urea four, diuretin three, and caffein, mercuric chloride, phyllicin, and ammonium chloride one each. Essentially the same type of curve was found in each experiment. In no instance did we observe any increase in the creatinine clearance following the administration of a diuretic. The clearance had no relation to the urine flow. On the contrary it either paralleled the level of the blood creatinine concentration or fell abruptly during the early part of the experiment to a more or less constant figure at which it remained during the latter part of the six-hour study.

Figure 2 shows the clearance value using the normal creatinine excretion as a basis for calculation. The half-hourly excretion as shown by the

blocked areas was practically constant. The blood creatinine concentration at the beginning of the experiment and after two hours was identical. Consequently, the clearance figures calculated from these two values were constant, even with a change in the urine volume of nearly ten-fold. This is exactly as one might have predicted. It has been repeatedly shown (Folin, 1905) (Marshall, 1920) that the normal excretion of creatinine is constant regardless of urine volume. Hence from the formula,

$$F = \frac{C\,u}{C\,b} \cdot V$$

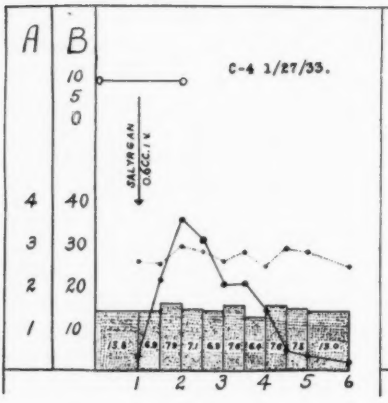

Fig. 2

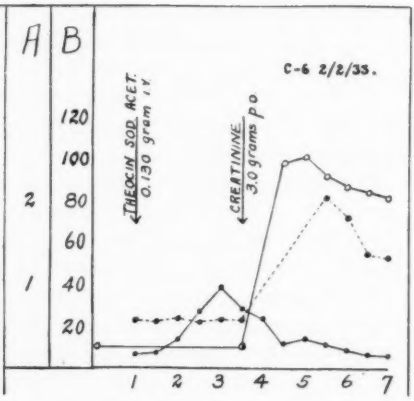

Fig. 3

Figs. 2 and 3. Blocked areas represent total creatinine excretion in milligrams.
Abscissa: Time in hours.
Ordinate: A. Urine volume in cc./min. ●——●——●
B. { Blood creatinine in mgm./liter ○——○——○
Creatinine clearance in cc./min. ●----●----●

it is readily seen that if the blood level is constant and the excretion does not change, the figure for creatinine clearance will likewise be constant. This was found to be true in twelve experiments in which the clearance was calculated from the normal excretion of creatinine.

Figure 3 shows the results of an experiment where both the normal and elevated blood levels were employed in estimating the creatinine clearance. The diuresis resulting from the injection of theocin-sodium-acetate was in no way reflected in the clearance values calculated from the normal excretion. After the influence of the diuretic had diminished, as manifested by decreasing urine volumes, 3.0 grams of creatinine were given by stomach tube. The blood concentration rose from 1.15 mgm. per

cent to 9.99 mgm. per cent after one hour. The creatinine clearances, which had been around 26 cc. per minute rose to 82, and fell abruptly to 55 cc. per minute with the fall in the blood creatinine concentration. Again there was no relation between the change in the creatinine clearance and the variations in the urine volume.

Shannon, Jolliffe, and Smith (1932) have suggested the use of the "true" creatinine values determined after the method of Gaebler (1930). We have made observations on the creatinine clearance using this procedure and have found that the creatinine values obtained from the Lloyd's reagent are 20 per cent to 30 per cent lower than the "normal values." There is a corresponding increase in the clearance calculated from these figures for "true" creatinine but the general shape of the curve is unaltered.

From the comparison of the clearance figures of xylose, raffinose, sucrose (given subcutaneously), and glucose (after phlorhizin poisoning) Jolliffe, Shannon, and Smith (1932) have postulated that these are true no-threshold substances, suitable for the determination of the glomerular filtrate. The ratio, creatinine clearance/xylose clearance in their later studies noted above showed an average value of approximately 1.3. This excess of creatinine they attributed to its "tubular excretion," a supposition further supported by the observation that this "tubular excretion" of creatinine was abolished after phlorhizin poisoning when the ratio of the two clearances became approximately one. We have made a few simultaneous determinations of the xylose and creatinine clearance using the technique described by these authors except for the administration of phlorhizin.[2] The ratio of the creatinine to the xylose clearance in these experiments was essentially the same as that reported by Shannon, Jolliffe, and Smith, averaging 1.23 in one such experiment of six observations.

COMMENT. The use of creatinine as a measure of glomerular filtration depends upon the acceptance of the premise that creatinine is excreted solely by filtration through the glomerulus and undergoes no reabsorption in the tubules. The direct observations of Bordley, Hendrix, and Richards (1933) show that creatinine exists in the glomerular urine of frogs in the same concentration as in the blood plasma, which indicates that this substance is filtered through the glomerulus. Evidence is lacking however that this is the sole method of excretion or that there is no tubular reabsorption. Inferences drawn from indirect evidence are conflicting, and the contradictory results from observations on the concentration ratios of creatinine, sulphate, phosphate, and glucose do not give a conclusive answer to the exact method of creatinine excretion.

The constancy of the creatinine excretion which occurs with a normal

[2] We wish to express our thanks to the U. S. Bureau of Standards, Washington, D. C. for the xylose, and to Standards Brands Inc., New York, N. Y. for the yeast used in this work.

creatinine concentration in the blood is illustrated in figure 2. This constancy has been found by others (Shaffer, 1908) (Hunter, 1922) to be uninfluenced by such factors as exercise, diet, diuresis, or disease. On the basis of the Rehberg theory, such a constant rate of creatinine excretion implies a constant rate of glomerular filtration. This seems unlikely in the light of the physiological variations which may influence the rate of filtration through the glomerular capillaries, such as changes in blood flow, blood pressure, the number of open capillaries, etc.

Certainly from our observations on the behaviour of the creatinine clearance after feeding creatinine, we may question the validity of Rehberg's assumptions. If the creatinine clearance is a measure of glomerular filtration, why do we find that an increase in the creatinine concentration in the blood causes an increased filtration rate at a time when the urine flow is at a relatively low level? Why should a decrease in the blood concentration cause a decrease in filtration, even during periods of diuresis?

Results similar to ours have been reported by Chrometzka and Unger (1931) who found in normal humans a constant fall in the creatinine clearance with falling blood levels. Such decreases were uninfluenced by various diuretics. The abrupt rise in the clearance values after the administration of creatinine is shown by the figures of Wyschegorodzewa (1931) who found increases of 100 per cent and more after giving creatinine to patients with renal disease. Stub (1932) reported higher initial values than were obtained subsequently. In the experiments of Rehberg (1926) one finds in three of the four tables, a drop in filtration as follows: 135 cc. per minute to 57 with a fall in the blood concentration from 7.75 mgm. per cent to 3.90 (table 1); 200 to 123 (table 3); and 129 to 111 (table 4). Fonteyne and Ingelbrecht (1923) studied the excretion of creatinine in the dog. Although their paper appeared in 1923, some years before the use of Rehberg's method to estimate glomerular filtration, the data necessary for such calculations are given. From their tables we have determined a rise in the creatinine clearance from 21.6 cc. per minute to 48.7 after the injection of 1.0 gram of creatinine intravenously. Subsequently, the fall in blood concentration from a maximum of 6.07 mgm. per cent to 1.85 mgm. per cent was accompanied by a fall in the clearance from 48.7 to 22.9 cc. per minute.

From our observations on the creatinine clearance in dogs and from the various objections raised by others, it appears unlikely that creatinine can be excreted solely by filtration through the glomerulus. We do not feel justified in offering as an alternative, tubular secretion although this view is widely held. (Chrometzka and Unger, 1931) (Marshall and Grafflin, 1932) (Clarke and Smith, 1932.) The reabsorption of creatinine or the addition of water by the tubules are by no means disproven possibilities. The exact mechanism of the excretion of creatinine remains undetermined,

and the conflicting results obtained from indirect observations cannot be explained on the basis of Rehberg's hypothesis. From this fact and the findings in our own experiments, the method of estimating the glomerular filtration by the determination of the creatinine clearance is a procedure of doubtful value

CONCLUSIONS

From these observations, the following conclusions may be drawn regarding the creatinine clearance in dogs:

I. The creatinine clearance for a given dog, calculated from the normal rate of excretion, is constant, regardless of marked changes in urine volume.

II. After feeding creatinine, the clearance rises and a subsequent fall in the blood concentration of creatinine is accompanied by a fall in the clearance value.

III. At the higher levels of blood creatinine concentration the creatinine clearance parallels the blood concentration.

IV. The changes in the creatinine clearance, reflecting the changes in the blood concentration of creatinine, bear no relation to changes in the urine volume as influenced by diuretic drugs.

V. These observations do not support the use of creatinine determinations in the blood and urine as a measure of glomerular filtration.

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THE INFLUENCE OF EPINEPHRIN UPON THE EXCHANGE OF SUGAR BETWEEN BLOOD AND MUSCLE[1]

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In a previous communication (1) the work of Bollman, Mann and Magath (2) and of others was confirmed and extended to show that neither epinephrin, ether anesthesia nor asphyxia has any effect upon the falling blood sugar level of abdominally eviscerated dogs. It was concluded that muscle glycogen is not an available source of blood sugar in the absence of the liver and that this organ, in the absence of food, is therefore the sole source of supply for the glucose in the blood. These results indicated that the hyperglycemia which follows the administration of epinephrin to normal animals is due to an accelerated release of blood sugar from the liver.

In a recent review, Cori (3) has marshalled evidence from which he concludes that epinephrin hyperglycemia is chiefly due to a decreased utilization of sugar by the muscles. This conclusion is based on three main theses:

I. The amount of glucose which had to be supplied to normal rats in order to produce a hyperglycemia of similar magnitude and duration as that caused by a certain dose of epinephrin, was such that it could not possibly be derived from preformed liver glycogen in the latter case.

II. Simultaneous determinations of arterial and venous blood sugar levels in men and in rabbits have shown that for the same degree of hyperglycemia, the arterio-venous blood sugar difference is greater when the hyperglycemia is caused by sugar administration than when it is due to epinephrin injection.

III. Colwell and Bright (4) have reported that when epinephrin and glucose are continuously injected into amytalized cats, the non-protein respiratory quotient (instead of rising progressively as under identical conditions without epinephrin) drops to the fat level within 3 to 5 hours, and remains there as long as the epinephrin administration persists. These

[1] Presented in preliminary form before the American Physiological Society, April, 1932.

[2] Aided by the Max Pam Fund for metabolic research.

animals excrete an increasingly greater proportion of the sugar administered and, in some animals, when glucose oxidation is presumably abolished, the sugar excretion tends to equal the supply for short periods of time. On the basis of these results, Colwell and Bright have concluded that the continuous intravenous administration of epinephrin—"suppresses glucose combustion even in the presence of an excessive supply, and may in fact completely abolish the ability to utilize glucose in any manner, as indicated by the ultimate total excretion of the supply."[3]

The comparison of the hyperglycemias following administration of glucose and epinephrin respectively as a method of estimating the glycogenolytic activity of the latter, seems to us unwarranted. It must be pointed out that Mann and Magath (5) have shown that it requires only about 250 mgm. of glucose per kilogram per hour to maintain the blood sugar level of the hepatectomized organism. This is only one-tenth of the amount of sugar which Cori injected into his rats in order to simulate an epinephrin hyperglycemia, and it seems obvious that the activity of the liver itself must be an important factor in the disposal of these larger amounts of sugar. But the activity of the liver following the administration of epinephrin and glucose respectively can hardly be considered to be similar. There is ample evidence that epinephrin causes the liver to pour out *some* sugar while, on the other hand, the administration of glucose probably causes a decreased glyconeogenesis and glycogenolysis in that organ (6). It is therefore reasonable to suppose that much more sugar would have to be administered, in order to produce a hyperglycemia of a certain degree, than would have to be released by the liver, under the influence of epinephrin, in order to produce a hyperglycemia of the same degree.

The present work was undertaken in order to test the 2nd and 3rd modes of evidence cited above. It occurred to us that the difference between the arterial and venous blood sugar levels could be used as a measure of sugar utilization of the muscles only if it could be assumed that the rate of bloodflow, and the hydration of the blood in its passage through the muscle, remained constant throughout the period of observation. It is obvious, for instance, that if sugar utilization remained unchanged and epinephrin were to double the rate of bloodflow through the muscles, the arterio-venous blood sugar difference would be halved. Similar variations

[3] It should be noted that Colwell and Bright have distinguished, very properly, between the "oxidation" or "combustion" of glucose, and its "utilization." This distinction is not so clear in Cori's review, since he has used the results of experiments dealing with each of these aspects as evidence for the other, without making sufficient reservations. In the present communication, the terms "utilization" or "retention" are used to indicate the sum total of the processes which operate in the disposal of sugar within the muscle, while "oxidation" is strictly reserved for that degradation process by which carbon dioxide and water are formed.

could result from concentration or dilution of the arterial blood during passage through the muscle. We accordingly determined the arteriovenous blood sugar difference in the hind limb of the dog, before and after the administration of epinephrin, and corrected these values for the observed changes in bloodflow and hydration.

With regard to the work of Colwell and Bright, whose conclusions were drawn from respiratory quotients and glycosuria, we attempted more directly to observe the passage of sugar out of the liver and into the muscles under similar conditions and for similar periods as in their experiments. Since technical difficulties precluded our use of a flowmeter on the liver

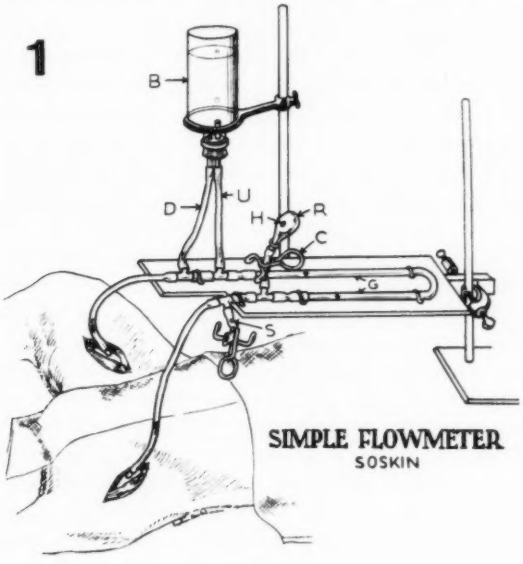


Fig. 1

or for very long periods on the muscles, we determined the blood-sugar levels simultaneously in the carotid artery and in the femoral, portal and hepatic veins, during prolonged administration of epinephrin.

We have found no evidence that epinephrin decreases the utilization of sugar by the muscle.

METHODS. The rate of bloodflow through the femoral vein, in the hind leg of amytalized and heparinized dogs was determined by means of a simple flowmeter, not hitherto described, which is shown in figure 1. This device consists principally of a bent pyrex glass tube, *G*, with an inside diameter of about 3 mm., calibrated so as to contain 2 cc. between

two marks which have been scratched upon it. The bore of the tube and the size of the calibration may be varied according to the range of flow rates which one expects to encounter. One end of the tube *G* is connected by gum rubber tubing to a pyrex glass cannula which receives the blood from the peripheral side of the ligated femoral vein of one hind leg. The other end of the tube *G* is similarly connected so as to deliver the blood into the central end of the ligated femoral vein of the opposite leg. The femoral artery of the latter limb is tied and cannulated, and from it the arterial blood samples are drawn. Venous blood samples are obtained from the side-tube, *S*. To make a flow reading, the rubber medicine-dropper bulb, *R*, is briefly compressed between the thumb and forefinger of one hand (the thumb being placed so as to occlude the hole, *H*, while, with the other hand, the spring clip, *C*, is momentarily released. This injects a bubble of air into the moving stream of blood in tube *G*. The passage of the air bubble between the calibration marks on tube *G* is timed with a stop-watch. At the distal end of tube *G* the air bubble passes upwards through the rubber tube, *U*, into the bubble collector, *B*, and displaces an equivalent amount of the heparinized physiological saline which this vessel contains downwards through tube *D*. When used in conjunction with known rates of flow, this flowmeter has been shown to be accurate within the limits of the smallest division of the stop-watch used.

"True blood sugar" determinations were made upon the simultaneously drawn samples of arterial and venous blood by the Somogyi modification of the Shaffer-Hartmann method (7). Total blood solids were determined by weighing before and after drying to constant weight in vacuum. The constant intravenous injections of epinephrin or epinephrin and dextrose were made with a simple constant injection apparatus previously described (8). The rate at which epinephrin was constantly injected varied from 0.06 mgm. per kgm. per hour, as in Colwell and Bright's experiments, to twice that amount. Such rates, as these authors have pointed out, lie well within maximal physiological limits.

The experiments in which the blood sugar levels were simultaneously determined in the carotid artery and the femoral, portal and hepatic veins were performed on amytalized dogs. One carotid artery and a femoral vein were cannulated. Blood was obtained from the portal and hepatic veins with a hypodermic needle and syringe, through an abdominal incision. The hepatic vein was approached between the liver lobes.

Amytal[4] anesthesia was used throughout these experiments because it was used by Colwell and Bright and by Cori, and because the latter author has concluded that this anesthetic acts synergistically with epinephrin to produce a complete inhibition of sugar oxidation.

[4] We are indebted to Eli Lilly & Company for supplies of sodium amytal.

RESULTS. Our results are illustrated by the experiments shown in table 1 and graphically represented in figures 2 to 6.

In experiment 15 (fig. 2) approximately equal increases in the arterial blood sugar levels were produced by the administration of 10 grams dextrose, and 8 grams of dextrose plus 0.25 mgm. epinephrin respectively. It may be seen that the presence of the epinephrin in the latter case has been followed by a greater rather than a decreased retention of sugar.

TABLE 1

EXPERIMENT NO.		TIME	BLOOD SUGARS			
			Hepatic vein	Portal vein	Femoral vein	Cartoid artery
		minutes	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*
54	Started constant intravenous injection of epinephrin—0.06 mgm. per kgm. per hour	0				
		15	105	134	104	117
		30	118	96	105	106
		70	87	94	88	81
		100	74	88	76	86
		130	65	78	81	88
		160	66	77	72	82
		190	102	77	70	87
		220	103	74	70	73
		250	102	70	69	84
		280	102	69	82	97
		300	110	69	72	70
58	Started constant intravenous injection of epinephrin—0.12 mgm. per kgm. per hour	0				
		5	150	121	121	129
		40	190	150	148	151
		70	192	129	133	156
		100	195	144	131	136
		135	148	123	132	135
		165	148	119	111	132
		200	183	147	125	143
		235	150	124	126	146
		270	154	96	109	120
		335	178	98	99	102

The algebraic sum of the areas above and below the zero sugar retention line, determined with a planimeter, total −77 for the hour following the dextrose administration and +200 for the same period following the injection of dextrose plus epinephrin. The control period preceding the first glucose injection shows the cyclic nature of glucose retention by the muscles, even when the arterial and venous blood sugar levels follow fairly smooth curves. It is apparent that the uncorrected arterio-venous

blood sugar difference gives but little insight into the exchange of sugar between blood and muscle. The marked negative phase following the rise in sugar retention caused by the dextrose administration, occurs in varying degrees throughout our experiments. In this experiment the administration of dextrose alone was followed by an increased rate of bloodflow, while the injection of dextrose plus epinephrin caused a slower

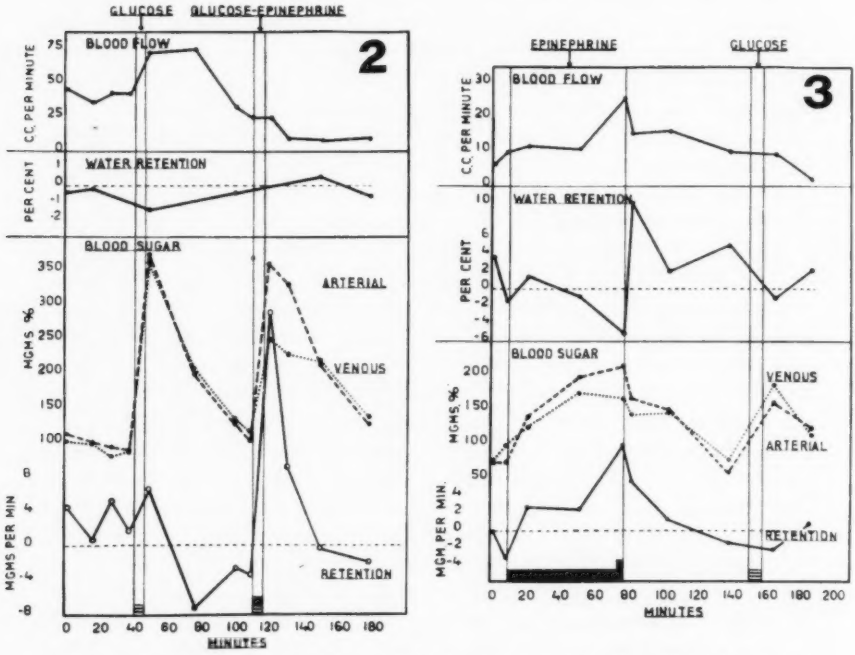


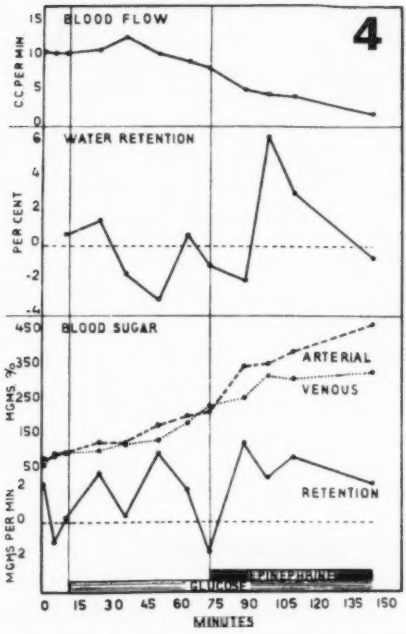
Fig. 2. Comparison of the effects of the intravenous administration of 10 grams dextrose, and of 8 grams dextrose plus 0.25 mgm. epinephrin respectively.

Fig. 3. Comparison of the effects of the constant intravenous injection of 0.12 mgm. epinephrin per kilogram per hour and the administration of 5 grams dextrose respectively. Where indicated, the rate of epinephrin injection was increased to 0.32 mgm. per kgm. per hour.

flow. The curve of water retention moves in an inverse direction to the rate of bloodflow, i.e., the more rapid the bloodflow through the muscle tissue, the less water it retains and vice versa. This also is the usual finding throughout our results.

Experiment 21 (fig. 3) is similar to experiment 15 except that the epinephrin was given alone, as a constant intravenous injection in physiological amounts. It may be seen that the sugar retention continued

throughout the period of epinephrin administration and increased as the amount of epinephrin injected was increased. This experiment illustrates rather well the inverse relationship between the curves of bloodflow and water retention. The cessation of epinephrin injection was followed by a sharp decrease in the rate of bloodflow through the muscles and an equally sharp increase in the water retention.



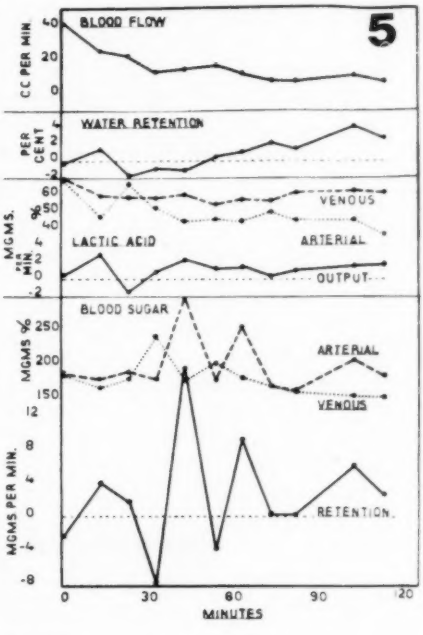


Fig. 4. Constant intravenous injection of 350 mgm. dextrose per kilogram per hour upon which there is later superimposed a constant injection of 0.06 mgm. epinephrin per kilogram per hour.

Fig. 5. Constant intravenous injection of 0.06 mgm. epinephrin per kilogram per hour which was started 4 hours and 35 minutes prior to zero time on graph.

In experiment 24 (fig. 4) a constant intravenous injection of epinephrin was superimposed upon a constant dextrose injection. The increased retention of sugar following the introduction of epinephrin is apparent. The planimeter readings for one hour of glucose injection and for the same period after the epinephrin was added, total +109 and +203 respectively. An interesting feature of this experiment is the fact that, although the arterio-venous blood sugar difference became progressively greater as the epinephrin administration continued, the correction for

bloodflow and especially for water retention caused the curve of sugar retention to assume a slightly downward course.

In view of the results reported by Colwell and Bright (4), experiment 26 (fig. 5) was performed to observe the retention of sugar by the muscles after prolonged epinephrin administration. Since technical difficulties prevented our use of the flowmeter for such periods, the animal was amytalized and epinephrin constantly injected for 4 hours, 35 minutes prior to the zero time of the experiment, and throughout the period of observation. The results of this experiment show that the retention of sugar by the muscles continues during the 5th, 6th and 7th hours of epinephrin administration when, according to Colwell and Bright, the low respiratory quotient

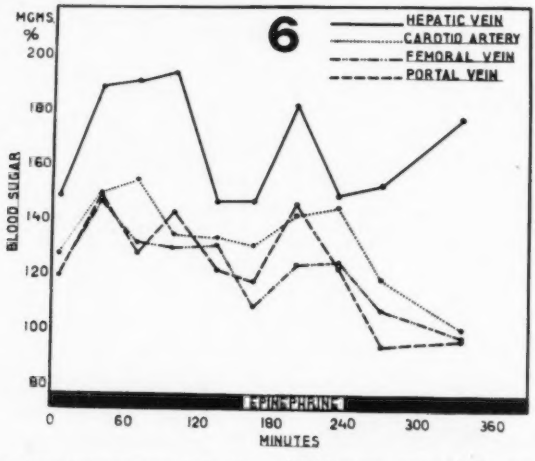


Fig. 6. Constant intravenous injection of 0.12 mgm. epinephrin per kilogram per hour.

and quantitative excretion of administered glucose presumably indicate the cessation of carbohydrate oxidation. The cyclic nature of sugar retention is well shown. The areas of the sugar retention curve above the zero line total +320, the areas below the zero line total −50, the algebraic sum being +270. This total is more than double the area representing the lactic acid output for the same period, which amounts to +115. As might be expected, the curve of lactic acid output is much smoother than that of sugar retention. It should be noted, for future reference, that the blood lactic acid levels remained at approximately constant heights throughout the two hour period of observation.

Experiments 54 and 58 are two experiments in which the blood sugar values of incoming and outgoing blood of muscle and liver were simul-

taneously determined during prolonged epinephrin administration. It may be seen that both these experiments confirm our flowmeter results, in that the blood sugar difference between the carotid artery and femoral vein indicates the retention of sugar by the muscle even during the 5th and 6th hours of constant epinephrin injection. The blood sugar values in the hepatic vein are of particular significance. In experiment 54 (table 1), where 0.06 mgm. of epinephrin per kgm. per hour was injected, the hepatic blood sugar level after falling below the other values during the first 3 hours, rose sharply during the 4th, 5th and 6th hours so as to indicate a pouring out of sugar from the liver during this time. In experiment 58 (fig. 6, table 1), where 0.12 mgm. of epinephrin per kgm. per hour was administered, the relatively high values at which the blood sugar level in the hepatic vein was maintained, can mean only that the liver was pouring sugar into the blood for the entire 6 hour period.

SUMMARY AND DISCUSSION. By the use of a simple flowmeter, it has been shown that epinephrin may either increase or decrease the rate of bloodflow through the muscles. The fact that both effects are observed may be due to the final effective concentration of epinephrin which reaches the muscles in a particular experiment. However, in view of the comparatively equal amounts of epinephrin used in the various experiments, it seems more probable that the dynamic results of epinephrin administration which we observed are a balance between its opposing effects upon the systemic blood pressure and the caliber of the muscle arterioles. The administration of dextrose usually increased the rate of bloodflow through the muscles. The exchange of water between the muscle tissue and the blood depends, in part at least, upon the rate of bloodflow. A decrease in the rate of bloodflow was usually accompanied by an increased retention of water in the muscles. An increased rate of bloodflow usually led to a decreased water retention or even a loss of water from the muscles. It is evident, from our results, that the uncorrected arterio-venous blood sugar difference can have no quantitative significance as regards the sugar utilization by the muscles. The passage of blood sugar into the muscle tissue, under all the conditions of our experiments, is a reversible and cyclic phenomenon. This applies to blood sugar as such, before alteration in the processes of storage or metabolism. It is not, therefore, incompatible with the conclusion drawn from previous work (1) to the effect that "once it has been deposited as glycogen in the muscles, glucose may be considered to have entered an irreversible reaction and cannot again appear as blood sugar in the absence of the liver." The cyclic nature of sugar retention makes it necessary to make numerous and frequent determinations of the arterio-venous blood sugar difference, even when corrections for bloodflow and hydration are being made. The frequent determination of the arterio-venous blood sugar difference, corrected for rate of bloodflow and hydra-

tion, has yielded no evidence that epinephrin decreases sugar utilization by the muscles.

The results of experiments 54 and 58 (fig. 6, table 1) in which epinephrin was constantly injected for periods as long as 6 hours, show that the liver continues to supply blood sugar and the muscles continue to retain it for such periods. In experiment 26 (fig. 5) the sugar intake by the muscles after prolonged epinephrin administration was quantitatively compared to the lactic acid output. As has been pointed out above, the output of lactic acid accounted for less than half of the sugar retained, while the level of the lactic acid in the blood remained relatively constant. This precludes the possibility that all the sugar removed from the blood by the muscles was returned to it in the form of lactic acid. It also indicates that a considerable portion of the sugar which disappeared must have originated in the liver from other source materials. The fact that these phenomena have been shown to occur at a time when, according to Colwell and Bright (4), the low respiratory quotient and the quantitative excretion of administered sugar supposedly indicate the complete cessation of glucose oxidation, shows that these criteria bear no necessary relationship to the amount of sugar which the muscles are utilizing. The present experiments cannot, of course, be interpreted in terms of carbohydrate oxidation. They do, however, lend greater validity to the alternative explanations for low respiratory quotients and quantitative sugar excretion which one of us (S. S.) has discussed, in connection with pancreatic diabetes, in a previous communication (9).

Our results and conclusions are supported by the work of Samson and Jacobs (10, 11) who found that prolonged constant injections (up to 13 days) of epinephrin in unanesthetized dogs yielded no evidence of a suppression of glucose utilization.

CONCLUSIONS

1. The arterio-venous blood sugar difference cannot be used as a quantitative estimate of sugar utilization by the muscles, unless it is corrected for rate of bloodflow and hydration of the blood, and determined at frequent intervals.

2. Whether or not the respiratory quotient of the entire organism and the degree of glycosuria can be used to determine the amount of carbohydrate oxidized by the muscles, these criteria bear no necessary relationship to the amount of sugar which the muscles utilize.

3. We have found no evidence that epinephrin decreases the utilization of sugar by the muscles.

We wish to acknowledge the assistance of the Department of Chemistry in the chemical analyses.

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THE ERYTHROCYTE COUNT, QUANTITY OF HEMOGLOBIN AND VOLUME OF PACKED CELLS IN NORMAL HUMAN SUBJECTS DURING MUSCULAR INACTIVITY

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In a recent study by Walters and Woodard (1933) in which day-to-day observations on the blood of normal human subjects were recorded for periods several weeks in length, significantly low erythrocyte and hemoglobin values were noted for which no explanation was found. Subsequent observations have demonstrated that when a period of muscular inactivity precedes withdrawal of a blood sample, the erythrocyte, hemoglobin and packed cell volume values are lower than the commonly accepted normal level. The purpose of this paper is to present a statistical comparison of the blood findings in normal human subjects after periods of muscular inactivity with those of normal individuals chosen at random with respect to physical activity, and to consider the possible explanations for the differences found.

METHODS. The subjects used in the study were men students between the ages of 20 and 30, all of whom had been found in good health by physical examination. During the periods of muscular inactivity, subjects rested on hospital beds in a quiet ward or room in the recumbent position. Resting samples of blood were withdrawn while the subjects were still lying supine.

Samples of blood were taken from an arm vein, 5 cc. being placed in a 15 cc. graduated centrifuge tube containing 1 cc. of 1.6 per cent sodium oxalate solution. One cubic centimeter was placed in a second tube with dry oxalate and was later used for making counts and hemoglobin determinations. Cell volume was ascertained by centrifuging for one hour at 2800 r.p.m. in a centrifuge with a working radius of 13.5 cm.

Erythrocyte counts were made with apparatus certified by the U. S. Bureau of Standards Trenner pipettes were used for dilution, a mechanical shaker for mixing, and Levy-Hausser chambers with improved Neubauer ruling for enumeration. Each erythrocyte figure recorded is the average of two counts from separate pipettes agreeing within 100,000 cells. Hemoglobin was estimated by the Newcomer method, the color disc used having been checked for accuracy by a series of comparative Van Slyke determinations.

The average volume, hemoglobin content and proportion of hemoglobin of the individual erythrocyte were obtained, the terminology and calculations suggested by Wintrobe (1929) being followed.

RESULTS. In order to compare the findings in resting subjects with those in non-resting subjects, blood studies were made of a group of individuals who were sampled as soon as they entered the laboratory at various hours of the day, no attempt being made to control the factor of previous activity. This group, consisting of subjects 1–80, will be designated in the present paper as series I. Complete data and a somewhat more detailed statement of procedure and precautions will be found in a separate paper (Walters, 1934).

A second group of 80 men was sampled after a one-half hour period of inactivity. This group, consisting of subjects 101–180, is designated as

TABLE 1

Comparison between mean values of series I and series II

	SERIES I SUBJECTS 1–80, SAMPLED AFTER RANDOM ACTIVITY, WITHOUT REST	SERIES II SUBJECTS 101–180, SAMPLED AFTER ½ HOUR OF INACTIVITY	DIFFERENCE
Erythrocytes	4,820,000	4,570,000	250,000
Total hemoglobin (gm. per 100 cc. blood)	15.17	13.74	1.43
Total volume of packed cells (cc. per 100 cc. blood)	46.3	43.8	2.5
Mean corpuscular volume (cubic micra)	96.2	96.1	0.1
Mean corpuscular hemoglobin (micromicrograms)	31.6	30.1	1.5
Mean corpuscular hemoglobin concentration (per cent)	32.6	31.4	1.2

series II. A comparison of the mean values found for these two separate groups of 80 each is given in table 1. From this tabulation it will be noted that the subjects of series II, after one-half hour of inactivity in the recumbent position, show lower values throughout than the subjects sampled at random.

A third group of 20 men was studied, first after random activity, subjects being sampled immediately upon entrance into the laboratory, then one hour later, after spending the intervening time in a state of muscular inactivity, lying in the recumbent position. This group, subjects 81–100, is designated as series III.

The data of this series show that the average erythrocyte count after an hour of muscular inactivity is 0.44 million lower than before lying down. A diminution in erythrocyte count is seen in all of the 20 subjects, the

greatest decrease being 780,000 cells. In quantity of hemoglobin, a decrease is seen in 16, no change in 3 and an increase in 1 of the 20 subjects. The average decrease is 1.05 grams and the maximum, 3.39 grams per 100 cc. blood.

All subjects except one show a decline in volume of packed cells after muscular inactivity. The mean difference after rest is 3.1 cc. and the maximum decrease is 5.5 cc. packed cells per 100 cc. blood. Rowntree, Brown and Roth (1929) found an average packed cell volume of 42.2 cc. in 49 normal men of various ages under basal conditions, compared with the mean of 43.8 cc. in series II and the resting mean of 44.3 cc. in series III.

In order to ascertain whether the decreases found are of sufficient magnitude to be other than of fortuitous origin, the statistical significance of each difference was calculated. For the difference between series I and series II, the following formula was applied (Dunn, 1929):

$$\text{Probable Error of Difference} = \sqrt{(PE_{\text{Mean I}})^2 + (PE_{\text{Mean II}})^2}$$

In the case of series III, the probable error of the difference was calculated by applying the usual formula for probable error of the mean to the difference between random activity and rest for each subject. A difference has been considered statistically significant whenever it is greater than four times its probable error.

Discussion. The difference between the subjects of series I and those of series II in erythrocyte count, quantity of hemoglobin and volume of packed cells is in each case too great, according to the statistical analysis, to be due to chance. This is also true of the same differences between the random and resting values of the individuals in series III.

A significantly lower figure is seen in mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration in the subjects of series II who were sampled after one-half hour of inactivity than in the random activity group, series I. Since no significant change occurred in the corpuscular values of series III, where the same subjects were examined both before and after rest, and since the corpuscular volume difference is negligible, the interpretation of this significant decrease is not clear.

The fact that the corpuscular values show no significant change in the observations of series III suggests that the alterations seen in total hemoglobin and total volume of packed cells are due to a decrease in the proportion of erythrocytes in the circulating blood rather than to any modification in individual cells. Two types of change could cause such a fall in red cell count: 1, an increase in plasma volume which would produce a relative decrease in number of cells, or 2, a redistribution of cells in the circulatory tract with the withdrawal of erythrocytes into storage depots, which would cause an absolute decrease of cells.

Increase in plasma volume. Thompson, Thompson and Dailey (1928) demonstrated a definite increase in plasma volume in patients assuming the supine position after having maintained the standing still position for some time. These investigators concluded that there is a loss of approximately 11 per cent of the total plasma volume due to capillary filtration when subjects continue the standing still position for 20 to 30 minutes, which is made up again in the same length of time after the recumbent position is assumed. These results were verified by Waterfield (1931) in a study of eight normal subjects. It is doubtful, however, that these findings are applicable to the subjects of series II and III, since their physical activity, though purposely left uncontrolled, consisted at least of a walk of 100 yards or more in most instances immediately preceding the period of recumbency.

Cassinis and Bracaloni (1930) found that the hydremic curve of blood varies less than 1 per cent during one to three hours of repose and also that in 40 to 130 minutes of walking the curve does not differ in any noteworthy degree from that of repose. This is in agreement with the work of Lee, Carrier and Whipple (1922) who observed that dogs exercised at a run for 20 minutes, although decreasing in weight as much as 12 ounces by fluid loss, maintained a constant plasma volume. Broun (1922) noted a slight increase of plasma volume in most of his dogs after short periods of exercise, but the variation averaged only about 3.6 per cent.

Priestley (1916) noticed that drinking 1 per cent sodium chloride solution produced a lowering of blood hemoglobin, although ingestion of water did not. Stebbins and Leake (1927) noted a slight fall in the specific gravity of human blood after the ingestion of food. Solarino and Cattaino (1930) observed in dogs and in man an erythropenia following administration of a meat meal or of hydrochloric acid having approximately the same concentration as that of gastric juice. The number of erythrocytes in the blood reverted to normal in three to four hours. Solarino (1931) found a corresponding augmentation in water content of the blood, which reached its height during the second hour of digestion. A cell decrease during digestion was also observed in rabbits by Raposo and Fevereiro (1931). Waterfield (1931), on the contrary, after determining blood volume in a subject, first in the fasting state, then after the taking of food, concluded that even a large meal has no appreciable effect on blood volume. Rud (1922–23) and Smith (1931) found that meals produced no significant change in erythrocytes.

Of the 80 subjects in series II, 61 were sampled between 2:00 and 2:30 p.m., or about one and one-half to two hours after the noon meal. The mean erythrocyte count in these subjects is 4.53 million. Fifteen subjects of the series were sampled between 3:00 and 3:30 p.m. or two and one-half to three hours after the noon meal. These subjects showed a mean count of 4.68 million.

It is difficult to tell what proportion of the 0.25 million average difference between the subjects of series I and II is due respectively to digestive effect and to the period of inactivity. That the latter is an important factor is indicated by observations on the subjects of series III, most of whom were sampled at about 10:00 and 11:00 a.m. Since all subjects attended 7:30 a.m. classes, this was approximately three and four hours after breakfast. Thus, during the fourth hour of digestion, when, according to Solarino, the erythrocyte count should have recovered from the digestive effect and returned to normal, a drop much larger than the difference between series I and II is seen.

TABLE 2

Statistical significance of decrease in circulating erythrocytes and hemoglobin following inactivity

	DIFFERENCE BETWEEN MEAN VALUES OF SERIES I, SAMPLED AFTER RANDOM ACTIVITY AND SERIES II, SAMPLED AFTER ½ HOUR OF INACTIVITY			DIFFERENCE BETWEEN MEAN VALUES OF SERIES III AFTER RANDOM ACTIVITY AND AFTER 1 HOUR OF INACTIVITY		
	Difference ± Probable Error	Diff. / P.E.	Significance	Difference ± Probable Error	Diff. / P.E.	Significance
Erythrocytes	250,000 ± 33,000	7.6	+	440,000 ± 29,700	14.8	+
Hemoglobin (gm. per 100 cc. blood)	1.43 ± 0.125	11.4	+	1.05 ± 0.139	7.6	+
Total volume of packed cells (cc. per 100 cc. blood)	2.5 ± 0.257	9.7	+	3.1 ± 0.262	11.8	+
Mean corpuscular volume (cubic micra)	0.1 ± 0.765	0.13	−	2.3 ± 0.801	2.9	−
Mean corpuscular hemoglobin (micromicrograms)	1.5 ± 0.309	4.9	+	0.6 ± 0.338	1.8	−
Mean corpuscular hemoglobin concentration (per cent)	1.2 ± 0.277	4.4	+	0.2 ± 0.281	0.7	−

Decrease in circulating erythrocytes. That muscular activity, even for short periods of time, results in notable increases in circulating erythrocytes has long been known. The older literature is well covered by the review of Hawk (1904) who also did considerable work on the subject, and his findings have been verified many times by subsequent investigators. The mechanism of the erythrocytosis produced by muscular activity is now fairly well understood.

The extensive researches of Barcroft (1931), of Binet (1930), and of Scheunert and Krzywanek (1927) have definitely established the spleen as a storage depot of blood cells, capable of increasing the oxygen-carrying

capacity of the blood by the extrusion of considerable quantities of erythrocytes. Although these investigators worked almost entirely with lower animals, the experiments of Benhamou, Jude and Marchioni (1929) with the effects of exercise and epinephrin upon splenectomized human subjects and those of Yang and Chang (1930) upon human subjects with splenomegaly indicate that the spleen performs a similar function in man.

According to Rein (1933), the normal organism has a tendency to maintain the lowest degree of circulatory activity consistent with its requirements at any given time, and will accordingly withdraw blood into storage areas whenever possible. The investigations of Cook and Rose (1930) are in agreement with this view. They showed that in cats recovery of the contracted spleen following muscular activity commences within one minute after cessation of the exercise.

The drop in erythrocyte count, hemoglobin and packed cell volume seen in the subjects of series III after a one-hour period of inactivity seems to represent an absolute decrease in the number of circulating erythrocytes, caused by the withdrawal of cells into storage depots, principally into the spleen, where Barcroft and Florey (1928) have shown concentration may occur through the drainage of fluid by lymphatics. Scheunert and Krzywanek (1927) failed to find any evidence that the erythrocyte storage function had been taken over by any other organ in dogs which had been splenectomized one year previously. The efforts of Benhamou, Jude and Marchioni (1929) to produce erythrocytosis were likewise negative in human subjects with splenectomy of as long as three and six years' duration.

The decrease in resting subjects probably represents the converse of the erythrocytosis which occurs in exercise, emotion, etc., both types of adjustment reflecting the delicate balance which at all times appears to adapt the oxygen-carrying capacity of the circulating blood to the requirements of the tissues.

SUMMARY

1. The erythrocyte count, quantity of hemoglobin and volume of packed cells in 80 healthy men aged 20 to 30 and determined after a one-half hour period of inactivity in the recumbent position, were compared with those of 80 other subjects sampled after random uncontrolled activity. Resting subjects showed a significantly lower red cell count, hemoglobin and packed cell volume.

2. Similar observations were made on 20 men, first after random activity, then one hour later after compiete muscular inactivity in the recumbent position. Red cell count, total hemoglobin and total volume of packed cells showed a significant decrease following inactivity while corpuscular volume, corpuscular hemoglobin and corpuscular hemoglobin concentration did not undergo any significant change.

3. The lower figures after inactivity indicate an absolute decrease in circulating erythrocytes due to withdrawal of cells into storage.

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AN INDIRECT METHOD FOR MAKING RED CELL COUNTS

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METHOD. The principle of the method is that the usual equation for finding the volume of a single red cell

$$\text{Volume of single cell, } \mu^3 = \frac{\text{Percentage volume}}{\text{Number of cells per mm.}^3 \times 10^{-7}}$$

can be arranged as

$$\text{Number of cells per mm.}^3 = \frac{\text{Percentage volume} \times 10^7}{\text{Volume of single cell, } \mu^3}$$

The value of the numerator of the right hand member of this equation can be found with sufficient accuracy by a suitable hematocrite method, and the value of the denominator from the diffractometric measurement of the radius of spherical red cells in lecithin-treated plasma. In this way the figure for the red cell count per cubic millimeter of blood can be found with sufficient accuracy without counting the cells at all.

The procedure used in practice is as follows. From 0.5 to 1 cc. of oxalated blood is required, and is obtained either from the finger, or, if a perfectly representative sample is required, by heart puncture in animals or venipuncture in man. A small quantity is introduced with a capillary pipette into a hematocrite tube 100 mm. long and 1 mm. in bore, and this is spun at 12,000 r.p.m. per 15 minutes, at the end of which time Koeppe's criterion is always observed. It is usual to make hematocrite determinations in duplicate, but this is unnecessary, for duplicates always agree very closely.

The remainder of the blood is centrifuged for about 3 minutes at 2,000 r.p.m., and from 0.2 to 0.5 cc. of plasma obtained. This is placed in a small agate mortar, and a fragment of "lecithin ab ovo" (Merck), about the size of a pin head, emulsified in it. To this lecithin-treated plasma is added a small drop of the packed cells from which the plasma was removed; the exact proportions of packed cells to lecithin-treated plasma is not very important, and is soon learned by experience. A small drop of the mixture is put on a slide, covered with a cover-glass, and examined under high power (dry). All the cells should appear spherical. The preparation is

then placed on the stage of the diffractometer (see Ponder and Saslow, 1931), and θ measured with respect to the junction of the blue and green bands of the 2nd order spectrum. The spectra ought to be very brilliant. If they are not, a fresh preparation is made. The mean volume of the single cell is then calculated from

$$V = \frac{4}{3} . \pi . (1.095 \text{ cosec } \theta)^3$$

Division of the figure for percentage volume $\times 10^7$ by the figure for V (obtained in μ^3 from the equation just given) provides the number of red cells per cubic millimeter of blood (see also Ponder, 1933).

This method has many advantages over the ordinary method of red cell counting. *a.* It is far more rapid when a number of determinations have to be made, for measurements of percentage volume can be made on as many as six samples simultaneously by using a hematocrite head carrying six tubes, and six diffractometric measurements of cell volume can be made almost as rapidly as one. In consequence the red cell counts for as many as six samples can be made in less than thirty minutes; accurate cell counts on six samples, by the usual method of counting on a hemocytometer chamber, would take, on the other hand, at least four times as long. *b.* The strain of counting individual cells on a hemocytometer is eliminated. *c.* Three important figures, viz., that for red cell count, that for mean cell volume, and that for percentage volume, are obtained simultaneously, and *d*, if a hemoglobin determination is done at the same time we also obtain values for mean corpuscular hemoglobin and for mean corpuscular hemoglobin concentration. And, lastly *e*, the values obtained are necessarily in absolute units. Those obtained by the hemocytometer are not necessarily so, unless the pipettes and chambers are very carefully calibrated.

In accuracy, the method is not inferior to that of red cell counting by the hemocytometer. Errors of packing in the hematocrite tube are substituted for errors of diluting, counting and random distribution, but the former are of about the same magnitude as the latter, as will be shown below.

Comparison of the method with the hemocytometer method. We have examined the accuracy of this new method in the following way. Red cell counts were made by two observers by the hemocytometer method, all the squares of 2 sides of 2 chambers being counted. The two observers (A. S. G. and M. D.) are both experienced counters, accustomed to work together, and usually able to check each other's results closely. Their counts, $\times 10^{-3}$, are shown in the first two columns of the table, the average in the third, and the percentage deviations of their respective counts from the average in the fourth (Δ_1, per cent). In the fifth column are shown

the counts as obtained by the indirect method now described, also $\times 10^{-3}$. The sixth column shows the deviation of this last figure from the average shown in column 3, the deviation being expressed as a percentage of the average (Δ_2, per cent). In the case of the figures for man, all the individuals were normal, but in the case of the figures for the rabbit some of the animals were anemic as a result of the withdrawal of blood by heart puncture. The extent of the anemia may be judged by the figures for the red cell count. Figures for the sheep are also included, to show that the method is not limited in its application to cells whose volume is comparatively great.

	1st count	2nd count	average	Δ_1, p.c.	indirect method	Δ_2, p.c.
Human	5,088	5,046	5,067	0 8	5,325	+5 1
	5,546	5,636	5,591	1 6	5,587	−0 1
	5,010	5,214	5,112	4 0	4,977	−2 6
	5,336	5,158	5,247	3 3	5,250	+0 1
	4,984	5,102	5,043	2 3	5,085	+0 8
Rabbit (normal)	6,852	6,634	6,743	1.7	6,886	+2 1
	5,566	5,474	5,520	1.0	5,260	−4.7
	6,922	6,810	6,866	1.7	6,964	+1.4
	5,910	6,082	5,996	1.4	6,175	+3.2
	7,280	7,422	7,351	1.9	7,143	−2.8
Rabbit (anemic)	3,304	3,276	3,290	0 8	3,232	−1.8
	3,440	3,654	3,547	1.4	3,724	+2.8
	2,380	2,592	2,486	8.5	2,766	+11 2
	3,882	3,982	3,932	2.5	3,933	0.0
	3,576	3,608	3,592	0 9	3,637	+1 2
Sheep	13,584	13,330	13,457	1 9	13,600	+1 1
	14,052	14,356	14,204	2 1	14,202	0 0
	15,044	15,286	15,165	1 6	14,880	−1 9
	14,320	14,196	14,253	0 9	14,510	+1 8
	15,250	15,420	15,335	1 1	15,307	−0 2

It will be observed that the discrepancies between the results obtained by the new indirect method and those obtained by the ordinary method of cell counting are of about the same magnitude as the differences between the duplicate counts. The new method sometimes gives a slightly higher value than does the hemocytometer method, and sometimes a slightly lower one; the discrepancy, however, is rarely as much as 5 per cent, which is very satisfactory considering that any such discrepancy is just as likely to be due to the hemocytometer method being in error as to the new method being inexact. The only cases in which we found fairly large discrepancies (e.g., 8.5 per cent and 11.2 per cent) were in anemic animals,

for, when the number of red cells per cubic millimeter is very low, neither the new method nor the hemocytometer method is as reliable as it is when the number is greater. In the case of the hemocytometer method, the decrease in precision is due to the distribution of a small number of cells being less perfect than that of a larger, while in the new indirect method the fall in precision is due to the measurement of percentage volume becoming less accurate as the number of cells decreases, and possibly also to the diffraction patterns being less brilliant because of the greater anisocytosis met with in anemic blood.

The new method is applicable, of course, only to cells which are rendered spherical by the addition of lecithin to the plasma surrounding them, i.e., to the cells of the mammalia, excluding the Camelidae.

SUMMARY

Since the number of red cells per cubic millimeter of blood is given by dividing the figure for percentage volume, $\times 10^7$, by the mean volume of the individual cell in μ^3, and since the percentage volume can be found by a high speed hematocrite and the individual cell volume by diffractometric measurement of the radius of the spheres which result when "lecithin ab ovo" is emulsified in the plasma surrounding them, the number of cells per cubic millimeter of blood can be found without counting the cells at all. The method, which is very rapid, is fully described, and it is shown that the results given agree with those given by ordinary red cell counting methods.

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THE EFFECTS OF POTASSIUM, COCAINE, URETHANE, AND ALCOHOL ON THE COLD BLOCK IN NERVE[1]

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As reported previously by Boyd and Ets (1934) two types of low temperature block can be obtained on the sciatic nerve of R. pipiens. One is the freezing block, seen usually in freshly excised or Ringer-soaked preparations, apparently affecting all the fibers nearly simultaneously. The other type of block develops above the ice-formation temperature, the muscle response most often showing a gradual diminution as the nerve is cooled. The latter block is found typically in nerves excised without application of saline solutions, and kept for some time in moist air or in paraffin oil. If these conditions are maintained for a sufficiently long period, the blocking temperature rises gradually until finally the nerve is inexcitable at room temperature. The nerve recovers in Ringer's solution, however, and with continued soaking conduction becomes possible at lower and lower temperatures until finally block can be produced only by freezing.

Frog muscle may also become non-irritable in moist air or in paraffin oil, and recover in Ringer's solution, as shown by Duliére and Horton (1929). These authors found that the inexcitable state develops rapidly in muscle after damage by pin-pricks. The early appearance of the non-freezing block (and later inexcitability) near the point of section in a nerve seems to be an analogous phenomenon.

Horton (1930) attributed the inexcitable state in muscle to an altered distribution of potassium between the inter- and intracellular fluids. He found that loss of potassium to the surrounding medium was necessary for the recovery of excitability, and that Ringer-soaked muscles became inexcitable when transferred to a solution containing an excess of potassium. For the latter effect loss of potassium from solution to muscle was not necessary. These findings suggest that the changes in irritability and conductivity of excised nerve, as mentioned above, may also be related to altered distribution of potassium. In histochemical studies, potassium is demonstrable in relatively high concentration near points of injury on

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a nerve (MacDonald, 1905; Menten, 1912). Overton (1904) showed that potassium salts may produce in nerve a state of reversible inexcitability. Apparently, however, no one has determined whether or not an actual transfer of potassium from solution to nerve is required for this effect. And while the behavior of nerve corresponds in several respects to that of the muscles studied by Duliére and Horton, a possible difference exists in regard to temperature.

There is no evidence that reversible inexcitability of muscle depends on temperature. Duliére and Horton (1929) did find that in July inexcitability was not obtained until the temperature was reduced to below 20°, but experiments in September did not confirm this, as inexcitability developed at 27°. They found that muscles which had become inexcitable at 20° remained so when warmed to 27°. These divergences they attributed to seasonal or other variations in the muscles used.

In nerve, on the other hand, the state of non-irritability (under the conditions described) is apparently reached by a progressive reduction of the temperature range within which conduction is possible. If altered distribution of potassium in the nerve trunk is responsible for the final loss of excitability, it is probably also the cause of the early impairment of conduction at low temperatures. We have therefore studied the cold blocking temperature of nerves which had been soaked for varying periods in modified Ringer's solution containing an excess of potassium. For comparison, we have made similar experiments on nerves treated with cocaine, urethane, and ethyl alcohol.

Procedure. Fresh muscle-nerve preparations from R. pipiens were used for each experiment, and no saline solutions were applied except as noted. The preparations were kept at room temperature (18°–25°) in paraffin moist chambers. In the floor of each chamber was a small pit holding about 5 cc. of solution, in which the nerve only was immersed.

The basic normal Ringer's solution used contained NaCl, 650 mgm., $CaCl_2$ 12 mgm. and KCl 14 mgm. per cent. In studying the effects of excess potassium we used two modified solutions, one containing four times and the other ten times the normal concentration of KCl (56 and 140 mgm. per cent). In these solutions the NaCl concentration was reduced so as to maintain a constant osmotic pressure.

For local cooling of the nerve, and for measurement of the blocking temperature, we employed the method described in the previous paper (Boyd and Ets, 1934). The method used permitted a considerable range of supercooling.

Results. Table 1A shows that for nerves kept in solutions with excess potassium, the cold blocking temperature rises with increased time of immersion. This effect takes place more rapidly as the concentration of potassium is made higher.

A considerable individual variation is seen in table 1A, and it was thought that this might depend in part on the original condition of the nerves. The preparations listed in this table were all taken from frogs which had been kept in a shallow pan of water in an incubator at 25°C. for 24 hours or more. Twenty-eight such preparations were tested immediately after dissection, without application of any saline solution. Of these, 11 blocked without freezing, at temperatures from −0.6° to −7.1°C.

TABLE 1

Effect of potassium Ringer's solution on blocking temperature

IMMERSION TIME	A. NERVES FROM FROGS KEPT AT 25°				B. NERVES FROM FROGS KEPT AT 5-9°			
	K 56 mgm. per cent		K 140 mgm. per cent		K 56 mgm. per cent		K 140 mgm. per cent	
	FB*	NFB	FB	NFB	FB	NFB	FB	NFB
minutes								
16–30	−6.3°		−5.6°					
31–45	−6.0		−5.5	−4.6	−5.9		−6.9	
46–60	−3.9	0.1		−5.8	−7.7		−7.6	
61–75	−5.0	−5.4		−3.0	−6.7		−6.5	−5.5
76–90	−4.8	−4.8		−0.3	−6.4			−3.2
91–105	−5.1	1.3		1.3	−5.9			−2.3
106–120	−5.8	−6.5		11.8	−4.9			N.I.
121–135		1.7		N.I.	−5.8			13.0
136–150		−1.3		9.3	−6.0			4.1
151–165		2.7		N.I.	−7.8			2.6
166–180		N.I.			−5.0			7.3
181–195		N.I.				−4.1		N.I.
196–210		N.I.				−4.5		
211–225						−2.2		
226–240						−4.7		
241–255						6.0		
256–270						−3.0		
271–285						0.1		
286–300						−0.2		
301–315						−3.0		

* FB, freezing block; NFB, non-freezing block; N.I., nerve not irritable though muscle responds to direct stimulation.

The remaining 17 blocked by freezing, at temperatures from −4.0° to −8.1°C. Supercooled nerves which are still conducting always cease to do so within a few seconds after ice formation begins. The limit of supercooling reached before freezing began varied from one experiment to another. Hence the overlapping of the temperature ranges in the above groups. A more uniform behavior was obtained with preparations from frogs stored for at least 24 hours at 5° to 9°. Of 34 such preparations, freshly dissected, all blocked by freezing, at −6.1° to −9.5°. The experi-

ments listed in the remaining tables were all carried out on preparations from cold-stored frogs.

Table 1B shows the effects of the potassium-Ringer solutions on these nerves. It is evident that they are more resistant to the action of potassium than are nerves from frogs kept in a warm environment. Non-irritability was not observed in any of this group after treatment with KCl 56 mgm. per cent (up to 5 hours) though the blocking temperature rose. The nerves in table 1A were rendered non-irritable after exposure to the same solution for 3 to 4 hours. It may be noted that this is approximately the minimum concentration of potassium which Horton (1930) found effective in producing non-irritability in frog muscle. Horton adds in a footnote that this critical concentration applies to muscles used in March and April, and that the figure is considerably higher for summer frogs. Our experiments were carried out from January to June, inclusive. Within this period no seasonal variation in behavior was noted. It may be, however, that the preliminary cooling of the frogs prevented such variations from being observed.

Because of the well-known synergism of potassium and cocaine in producing nerve block, we were interested in finding out whether or not cocaine would affect conduction at low temperatures in the same manner as potassium. The effect of 1/1000 cocaine in Ringer is shown in table 2, and it is evident that with increased time of immersion the blocking temperature rises until the nerve is non-irritable. Nerves immersed in 1/2000 cocaine-Ringer, up to 75 minutes, blocked by freezing.

Similar experiments were carried out with urethane and with ethyl alcohol. The effect of 2 per cent urethane in Ringer is shown in table 2. It is somewhat similar to that of cocaine, although no blocking temperatures above zero are recorded. With 3 per cent urethane non-irritability was produced in 35 minutes.

Nerves treated with 10 per cent alcohol in Ringer became non-irritable in 15 minutes, but on removal from the solution and mounting in the cooling chamber they usually recovered within 4 to 6 minutes. They then blocked by freezing. The same result was obtained on nerves soaked in the alcohol-Ringer for periods up to 95 minutes. Recovery in air is so rapid that no effect of alcohol on the blocking temperature can be shown by our procedure. Rapid recovery after alcohol block is seen in the experiments reported by Lucas (1913).

The changes in blocking temperature and final non-irritability, shown in the tables, are not due simply to the gradual deterioration of the excised nerve. Nerves were kept in normal Ringer's solution for periods up to 5 hours and 20 minutes. These all blocked by freezing. Moreover, the rise in blocking temperature, in nerves treated with any of the agents listed, is a reversible process. Even after non-irritability has developed,

the nerves recover as a rule if transferred to normal Ringer's solution, and if kept there for a time can be cooled to freezing before block takes place.

DISCUSSION. It has been shown that the inexcitable state produced in nerves by treatment with excess potassium is preceded by a gradual impairment of conduction at low temperatures. In this respect it resembles the inexcitability which develops (most rapidly near the point of section) in nerves kept in moist air or in paraffin oil. Our observations do not prove that the latter condition is caused by altered distribution of potassium. They do show that the relation between temperature and the inexcitable state in nerve is consistent with the potassium hypothesis. Definite proof must apparently depend upon studies of the potassium

TABLE 2

Effects of cocaine and of urethane on cold blocking temperature

The nerves were all taken from frogs kept for 24 hours or more at 5 to 9°C. FB, freezing block; NFB, non-freezing block; N.I., nerve not irritable though muscle responds to direct stimulation.

TIME OF IMMERSION	0.1 PER CENT COCAINE-RINGER	2.0 PER CENT URETHANE-RINGER
minutes		
11–20	−7.7 NFB	−7.9 FB
21–30	−2.0 NFB	−7.8 FB
31–40	4.7 NFB	−5.0 NFB
41–50	6.7 NFB	−7.9 FB
51–60	N.I.	−7.6 NFB
61–70	N.I.	−3.2 NFB
71–80		−0.6 NFB
81–90		−4.3 NFB
91–100		−1.1 NFB
101–110		N.I.
111–120		N.I.

balance for nerve, similar to those made by Horton (1930) on muscle. Feng (1933) has related the reversible loss of tactile impulses from injured frog skin to the liberation and removal of potassium.

It is perhaps of some general interest that, in the course of action of potassium on the excised nerve, block occurs first in a locally cooled segment, while preliminary cooling of the animal increases resistance of the nerve to the same agent. The mechanism by which potassium or cold produces a block in nerve is not definitely known. The question is discussed by Gerard (1932, pp. 511–12) with references to a number of papers dealing with membrane changes and associated loss or return of conductivity under the influence of salts and polarizing currents.

Gasser (1931) studying the effects of temperature on nerve, found that

with cooling the temperature coefficient for the refractory phase increases, so that at the lowest temperature the refractory phase shows a gross prolongation. In preparations from frogs acclimated to 30°C., conduction stopped at plus 5.0°C., with complete recovery on re-warming. In the electrical response he found that cooling prolongs the spike but shortens the after-potential (also noted by Gasser and Erlanger, 1930). Graham (1933) finds that excess potassium produces a prolongation of the refractory period, and a reduction in magnitude and duration of the after-potential (also a reduction of the spike, though to a lesser degree). In view of these similarities in the effects of cold and of potassium, a synergistic action in producing nerve block might perhaps have been anticipated. Graham (1930) finds that urethane also affects the electrical response in somewhat the same manner as potassium.

Forbes and Thacher (1920) report that nerves from winter frogs, as observed at room temperature, show action potentials of long duration; and Gasser and Erlanger (1930) found that this was because of the long after-potential in nerves from winter frogs. The latter authors also state that if winter frogs are kept for a week at laboratory temperature the summer values of the after-potential are restored. The well-developed after-potential in the nerves of cold-stored frogs may be of some significance in explaining the high resistance of these preparations toward potassium and low temperatures.

SUMMARY

1. Immersion of the frog sciatic nerve in Ringer's solution containing an excess of potassium (56 mgm. or more per cent) raises the temperature at which cold block occurs.

2. This effect takes place with greater rapidity as the concentration of potassium is increased.

3. Nerves from frogs which have been kept in a cold environment show a relatively high resistance to potassium, as compared to nerves from warm-stored frogs.

4. Cocaine and urethane, in proper concentration, produce an effect on the cold blocking temperature similar to that of the potassium ion.

5. Alcohol (10 per cent in Ringer's solution) did not affect the blocking temperature in any way that could be shown by our procedure.

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THE EFFECT OF INJECTION OF AMNIOTIN ON THE SPONTANEOUS ACTIVITY OF GONADECTOMIZED RATS

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It is now a well-established fact that the general spontaneous activity of gonadectomized animals is much below normal. Wang (1923) found that ovariectomy in rats produces a great permanent decrease in their running activity as measured in a revolving drum. Hoskins (1925) reports a similar large decrease in adult males following removal of the testes. Moreover, animals of both sexes gonadectomized at an early age are less active than normal (Richter, 1933).

The activity of these sluggish gonadectomized animals can be markedly increased, sometimes to normal, by implantation of ovaries or testes. Wang, Richter and Guttmacher (1925) have shown that implantation of ovaries into the rectus muscle of castrated males or females increases the activity from the low castration level of only a few thousand revolutions to over ten thousand revolutions per day. Richter and Wislocki (1928) have found that implanted testes have a similar effect, although less certainly, due to technical difficulties connected with the grafting operation.

It is clear from these experiments that the gonads contain some substance which is necessary for the maintenance of a normal amount of spontaneous activity. The nature, site of production and mode of action of this substance, however, are not known. Attempts have been made to study the effects produced on the activity of castrated animals by extracts of the gonads. Bugbee and Simond (1926), working with one spayed rat, found that an extract made from the follicles of pigs injected daily for several weeks caused a marked increase in activity. At the same time Wang (unpublished), working in the Phipps Laboratory with a large number of animals, also found that the activity of spayed females could be greatly increased by injection of follicular extracts. The increase persisted as long as daily injections were given, but due to difficulties in obtaining good extracts at that time, the effects of the injections were very irregular and inconsistent and did not permit a quantitative study to be made.

The ready availability in the past year of large amounts of consistently potent extracts of oestrin in the form of amniotin (Squibb) has made it

possible to study in greater detail its effect on the activity of gonadectomized animals. Normal as well as gonadectomized male and female rats were used in the experiments reported below.

The writers wish to acknowledge their indebtedness to E. R. Squibb and Sons for the liberal supply of amniotin necessary to carry on these studies.

METHODS. In these experiments the activity of the rat was measured in terms of the revolutions made per day in a revolving drum. The technique has been described fully elsewhere (Richter, 1927). At the same time records were obtained of the daily food and water intake; vaginal smears were made each day; and the animals were weighed once weekly.

The problem was attacked from several points of view. In the major experiments twenty-nine young rats, fourteen males and fifteen females, were placed in the running cages at the usual age, between 35 and 45 days. When at 50 to 65 days of age they reached a good running level (about 7,000 revolutions per day) they were gonadectomized. Daily injections of oestrin were started on the day following gonadectomy in nine females and eight males. By starting the injections immediately after the operation we hoped to avoid atrophic changes and give the female hormone the most favorable opportunity for action. The other twelve animals were used as untreated controls.

In another experiment seven animals were gonadectomized at the early age of 1 to 10 days, and daily injections of sex hormone (oestrin) were started when the rats were placed in the running cages at 30 days of age. As a basis of comparison, average curves were used from a previous study on the effect of early gonadectomy on running activity.

An effort was made to reproduce the normal four-day cycle in twenty-nine gonadectomized males and females by injections every fourth day.

Finally the effect of amniotin injection on the activity of five normal animals was studied. Each animal received a total of 5 rat units of amniotin per day, in two doses, except on Saturdays and Sundays when the 5 units were given in one injection.

RESULTS. A. *Effect of amniotin injections on the activity of female rats spayed after maturity.* The effect of amniotin on the activity of sluggish spayed females may be seen in figure 1, A and B, in which the average daily activity for ten day periods in revolutions of the drum is given on the ordinates and the age of the animals in days on the abscissae.

Records of six females spayed at approximately 58 days of age, but untreated, are presented in figure 1 A. It can be seen that the activity of all of these animals definitely decreased after removal of the ovaries, so that it averaged less than 2,000 revolutions per day at the end of the experimental period.

In striking contrast are the records in figure 1 B of the seven spayed

animals which were treated with 5 R. U. of amniotin per day. These animals became increasingly active until after 50 days they had reached high running levels, in one instance almost 24,000 revolutions daily.

That amniotin caused the spayed females to become as active, if not more active than the normals is evident from figure 3 A. There the composite curve of the seven injected animals follows the normal curve (based

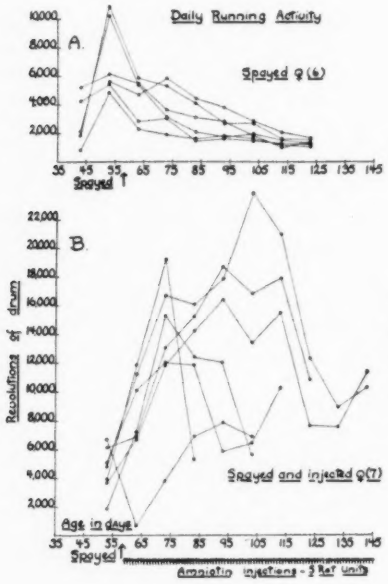

Fig. 1

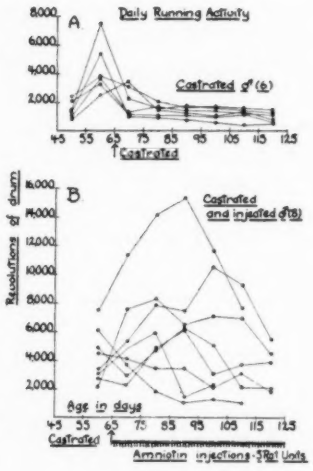

Fig. 2

Fig. 1 A. Individual activity curves (ten-day averages) of six females spayed at 58 days of age; untreated.

B. Individual activity curves (ten-day averages) of seven females spayed at 58 days of age; treated daily with amniotin.

Fig. 2 A. Individual activity curves (ten-day averages) of six males castrated at 65 days of age; untreated.

B. Individual activity curves (ten-day averages) of eight males castrated at 60 days of age; treated daily with amniotin.

on 19 females—Richter, 1933) very closely for 15 days after which it maintains an even higher level for several weeks. A composite average curve for the 6 untreated spayed females is also shown. The difference in activity of treated and untreated groups is very striking.

It appears, then, that so far as activity is concerned in females, the ovarian secretion may be replaced completely by daily injections of oestrin.

B. *Effect of amniotin injections on the activity of male rats castrated after maturity.* That oestrin has a very definite effect on the activity of castrated males, is apparent in figure 2 A and B. Individual curves for six untreated castrated males and seven castrates injected with amniotin, show that treated males are very much more active than untreated animals. Indeed in one instance a male reached the high level of the most active females.

In figure 3 B one sees that although the average activity of treated males is definitely higher than that of the untreated group, it is still considerably below that of the normal average (based on 19 normals, Richter, 1933).

It would seem from the above that the testicular secretions also can be replaced to a great extent by the female sex hormone.

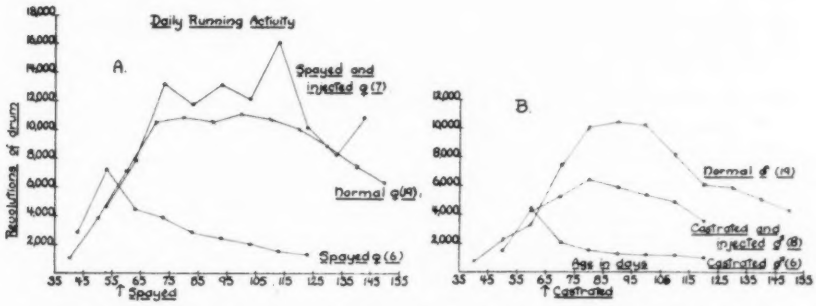

Fig. 3 A. Daily activity curves (ten-day averages) of nineteen normal females, six gonadectomized females, untreated, and seven gonadectomized females treated daily with amniotin.

B. Daily activity curves (ten-day averages) of nineteen normal females, six castrated males, untreated, and eight castrated males treated daily with amniotin

C. *Effect of amniotin injections on the activity of females spayed shortly after birth.* The results of these experiments are summarized by the curves in figure 4 A. The average curve taken from a previous experiment on 7 females spayed at 1 to 10 days of age is shown at the bottom. The group average did not rise above 2,000 revolutions per day, which is about $\frac{1}{5}$ as great as that of the normal females shown at the top. In marked contrast, the curve for the females, which received daily injections of amniotin from the age of 35 days on, closely approximates the normal. Attention must be called to the fact, however, that after 40 days of treatment the curve tends to fall below the normal, a fact which would seem to indicate that the daily injections did not fully replace the normal ovarian secretions.

D. *Effect of amniotin injections on the activity of males castrated shortly*

after birth. In these experiments the rats castrated at an early age (1–10 days) received daily injections of amniotin for 50 days. The results presented in figure 4 B show that whereas the untreated castrated rats remained very inactive, not making above 2,000 revolutions per day, the treated animals averaged over 7,000 at one time. Comparison of the curves for the treated animals and for the 19 normals shows that the treated castrates actually were more active than the normals for the first 30 days (i.e., up to the age of about 60 days). After that, however, the activity fell off until, at the end of the experiment 28 days later, the treated animals were considerably less active than the normals, although still much more active than those untreated.

There is no adequate explanation at hand for this failure to maintain a normal amount of activity; perhaps it was due to the fact that the dosage was not increased as the animal grew older. It is clear, however, that the hormone produces as great an effect on the early castrated males as on the early spayed females.

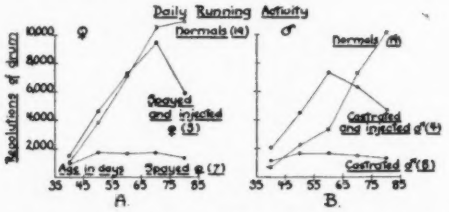

Fig. 4. A. Average daily activity curves of nineteen normal females, seven females spayed at 1 to 10 days of age, untreated, and three females spayed at 1 to 10 days of age and injected daily with amniotin. B. Average daily activity curves of nineteen normal males, eight males castrated at 1 to 10 days of age, untreated, and four males castrated at 1 to 10 days of age and injected daily with amniotin.

The greater effect shown above on the females at a later age, 60 to 80 days, must be due to sex differences which are built up by that time but which are not present at the ages from 1 to 10 days. One example of such a sex difference to account for differences in the effect of the extract on activity would be body weight; for at 1 to 10 days the males and females weighed about the same, but by 60 days the males weighed much more.

E. *Characteristics of the activity produced by daily injections of oestrin over long periods of time.* Inasmuch as the conditions of the experiment were kept very constant from day to day, it might be expected that the activity also would be almost constant from day to day. Such was not the case. In all but one or two animals there were great fluctuations and marked irregularities.

Since it would be of importance if daily injections of oestrin brought out the typical four-day sex rhythm in spite of the absence of the ovaries,

the charts of all of the treated animals were closely examined for the presence of such a cycle. One female gave a record which at first glance has much the same appearance as that of a normal female. The characteristic regular fluctuations were suggested and many of the intervals between peaks actually were 4 days in length. Thus, from the 50th day on, the intervals were 4, 3, 3, 3, 5, 4, 4, 2, 4, 5, 3, 3, 4, 3, 4, 3, 5, 4, 9, 2, 4, 6. The variation from 2 to 5 days is sufficiently great, however, to make it seem very unlikely that we are dealing here with the usual cyclical changes. It would seem more probable that these four-day periods simply mark chance fluctuations in this animal.

This view is borne out by the changes in the reproductive tract as studied from the vaginal smears, which contained many cornified cells every day and practically no epithelial cells or leucocytes. There was absolutely no indication of a four-day cyclical change in the smears.

Further study of the activity curves reveals a feature which is particularly worthy of note, namely, the presence of a long cycle with a duration of 80 to 100 days. Thus, in the normal female charts, in figure 3 A, it can be seen that the activity increased sharply until the animals were about 70 days old, after which it remained constant for about 50 days. From then on it decreased fairly rapidly so that at 150 days it was down to about 6,000 revolutions per day. The records of normal males, in figure 3 B, shows this feature even more strikingly.

This fall in activity after 120 days we are inclined to attribute to a lack of adjustment of the dosage of the hormone to the increasing size of the animals. If this is the case, one could not speak of a "cycle" in connection with the phenomenon. However, since a similar cycle is observed in normal animals, it is likely that we are dealing here with a real cycle conditioned by some mechanism independent of the gonads.

F. *Simulation of four-day cycles by injections every fourth day.* In order to test further the relationship between the sex hormone and the cyclical manifestations of activity a fairly large number of rats (eighteen females and nine males) were injected with 5 R. U. every fourth day instead of every day, with the idea of determining whether the normal four-day activity cycle could be set up in this way.

Fairly regular four-day cycles were found in seven of the eighteen females, and in two of the nine males, the peak of activity being reached regularly on the day following the injection.

A record from a castrated male also showed the four-day cycle but, due to the low level of activity, less strikingly. In this animal the peak of activity occurred on the second day after the injection. The four-day cycle then can be set up in both males and females by injections of oestrin every fourth day, but for some reason it is not possible to bring it out as consistently as might be expected even in females. The reason for this may again have to do with the dose of hormone given.

G. *Effect of injection of oestrin on the activity of normal animals.* Inasmuch as oestrin injected into spayed females returned the animals to average or even higher levels of activity we endeavored to determine whether injections might also increase the activity of normal animals. Accordingly the extract was given to three normal females and two normal males.

Injection of amniotin caused an immediate drop in activity which lasted about 15 days in all of the animals. After that the activity returned rapidly to its former level, but did not go beyond. It is of particular interest that the males as well as the females continued to run very actively after this interval of inactivity.

AUTOPSY FINDINGS. At autopsy the uteri and mammary glands of the spayed females presented, grossly, a normal picture. The uterine horns were never atrophied; on the other hand, at the end of the persistent injections the horns were either a little enlarged or in the dioestrous condition.

In marked contrast the castrated males showed the typical castrate condition of the sex organs. The penis was atrophied, the prostate gland and the seminal vessicles were either extremely small or absent to ordinary inspection and the glands were not demonstrable.

REMARKS. The above experiments indicate that the oestrin contained in amniotin, injected into spayed females, almost completely replaces the secretion from the ovaries; the sex apparatus is normal and shows no atrophy and the animals are fully as active as normals, some of them even more active.

The results obtained by injection of the female sex hormone into castrated males are of particular interest since the hormone has a marked effect on activity even though it has no effect at all in preventing the usual castrate atrophy of the seminal vesicles or the prostate gland.

The conclusion to be drawn from these observations is that the gonads contain some substance which is specific for activity, and independent to a great extent of the substance which controls the growth and development of the sex apparatus. Were chemists able to isolate such a substance it might be of use in the treatment of some of the conditions of inactivity seen in psychiatric clinics.

SUMMARY

1. Oestrin (5 rat units daily) injected into seven spayed female rats maintained not only a normal state of the reproductive tract but also a normal level of spontaneous activity. Untreated spayed animals, on the other hand, showed atrophic changes in the tract and became very inactive.

2. Eight castrated males treated with the hormone experienced a similar,

though less marked, increase in activity. This effect occurred despite the involution of the male secondary sex glands.

3. The same result was obtained in seven animals gonadectomized at early ages (1 to 10 days) and injected first at 35 days of age.

4. Inasmuch as oestrin produced a marked increase in the activity of gonadectomized males without preventing the castrate atrophy of the sex apparatus, it is concluded that the hormone contains a factor specific for activity and independent at least in large part of the sex factor.

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THE EFFECT OF FLUID DEPRIVATION AND FLUID INTAKE UPON THE REVIVAL OF DOGS FROM ADRENAL INSUFFICIENCY[1,2]

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In the course of a study of blood pressure, hemoglobin concentration, and blood urea nitrogen changes, in bilaterally adrenalectomized dogs exhibiting severe symptoms of insufficiency and following revival of the animals by injections of cortical hormone, it was repeatedly observed that the dogs showed remarkable recovery towards normal, despite the total lack of fluid or food intake. Although the adrenalectomized dog exhibiting symptoms is apparently unable to dilute his blood and thereby restore the diminished volume of fluid in circulation, yet within a few hours after injection with adequate amounts of cortical hormone, the same dog exhibits marked dilution (Swingle, Pfiffner, Vars, Bott, and Parkins, 1933). The only fluids available for mobilization and transfer are those present in the animal's body at the time of injection. The experimental data at present indicate that the chief rôle of the adrenal cortical hormone in the mechanism of blood dilution and recovery from insufficiency, is probably that of a mobilizer of fluid (and salt; Loeb et al., 1933; Harrop, Weinstein et al., 1933) in the tissues and interstitial spaces for transfer to the blood stream.

The following experiments were undertaken with the view of determining how far along the road to recovery the injected animals can go in the total absence of any food or fluid intake. The type of experimental animal employed and the methods used for blood pressure (abbreviated to Bp. in the protocol), hemoglobin (Hb. in the protocol), blood glucose and urea nitrogen are identical with those referred to in earlier papers (Swingle, Pfiffner, Vars and Parkins, 1934; Parkins, 1934). The dogs were permitted to develop severe symptoms of insufficiency by withdrawal

[1] In the collaborative studies on the adrenal cortex carried on in this laboratory the writers have specialized in certain phases of the experiments. The physiological aspect of the problem is the responsibility of the senior author (W. W. S.); the biochemical work of extraction and purification of the hormone that of one of us (J. J. P.).

[2] The expenses of this investigation have been aided by a grant from the Josiah Macy, Jr. Foundation, New York.

of cortical extract for a few days, and when they were verging on prostration they were injected repeatedly with large doses of highly potent hormone, but all food and water were withheld.

Eight animals were employed in the experiments; results were similar in all cases. The sample protocol and graph recorded are representative and give the pertinent data.

Protocol 1. Dog 130. Adult male. Bilaterally adrenalectomized 9/3/33. Maintained in good health[3] by adequate doses of cortical extract. Injections discontinued 10/27/33. Pulse 70, Bp. 96, Hb. 11.5, urea 21, weight 12.3 kgm. On 11/4/33 at 12:55

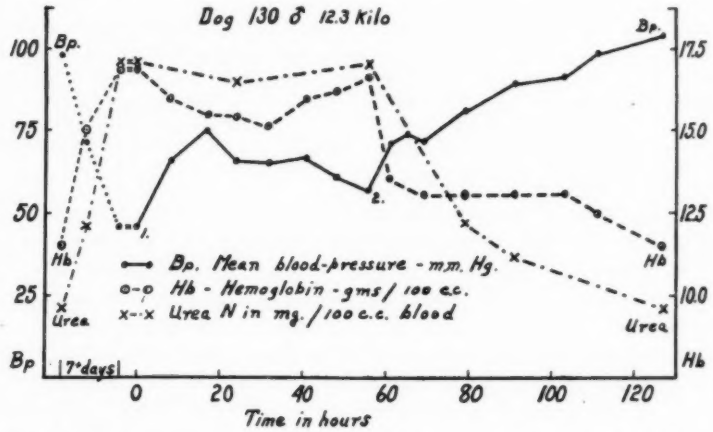

Fig. 1. Blood pressure, hemoglobin and urea nitrogen changes in an adrenalectomized dog (exhibiting marked symptoms) receiving repeated large doses of cortical hormone but deprived of food and water. Seven days elapsed from time of extract withdrawal to first injection. *1* = first extract injection. All food and water were withheld during the 56 hour interval between points *1* and *2* on the graph. *2* = dog permitted to take food and water. Note the inverse relation between hemoglobin and urea, and the blood pressure. Left hand scale, blood pressure and urea; right hand scale, hemoglobin. Protocol 1.

a.m. dog exhibited symptoms of insufficiency; he was weak and spastic and had not taken either food or water all day. Pulse 60, Bp. 46, Hb. 16.8, urea 96. All food and water withheld. Animal injected intravenously with cortical hormone (3 cc. per kgm.). At 9 a.m. pulse 124, Bp. 66, Hb. 16. Dog injected with 17 cc. of extract intraperitoneally. At 6:10 p.m. pulse 112, Bp. 75, Hb. 15.5. Animal passed 240 cc.

[3] The Hb. of bilaterally adrenalectomized dogs is generally, if not invariably considerably lower than normal. The experimental details relating to this observed anemia will be presented elsewhere. Suffice it to state here, that our data indicate that the lowered Hb. is due to loss of the adrenals, and not to dietary or other disturbing factors.

urine. 11/5/33 at 1:30 a.m. pulse 132, Bp. 65, Hb. 15.4, urea 90. Dog given 16 cc. extract intraperitoneally. At 9:20 a.m. pulse 140, Bp. 65, Hb. 15.1, weight 10.9 kgm. Animal lively and playful but shows signs of weakness when running about. Sixteen cubic centimeters extract injected intraperitoneally. On 11/6/33 at 10:10 a.m. pulse 148, Bp. 61, Hb. 16.2. Animal passed 130 cc. urine. Dog playful and lively but signs of weakness present. Seventeen cubic centimeters of extract injected intraperitoneally. At 9:15 a.m. animal less lively and playful. Pulse 140, Bp. 57, Hb. 16.6, urea 95, weight 10.6 kgm. Dog had passed 95 cc. of urine. Three hundred grams of ground beef plus 200 grams of Ken-L-Ration placed in cage along with 1000 cc. of water. Animal refused the food but drank 360 cc. water and by 10:15 a.m. he took an additional 200 cc. At 11:45 a.m. pulse 132, Bp. 72. Given 17 cc. extract intraperitoneally. Dog urinated 350 cc. and had by this time taken a total of 900 cc. of water since 9:15 a.m. On 11/7/33 at 9:30 a.m. pulse 104, Bp. 82, Hb. 13.1, urea 47. Animal bright and lively. No symptoms. Dog had taken a total of 1100 cc. water Urinated 225 cc. during night. At 9:30 p.m. pulse 108, Bp. 89, Hb. 13.1, urea 36. Animal active and vigorous. The dog urinated 215 cc.; drank 360 cc. water; ate 360 grams ground beef. On 11/8/33 at 9:30 a.m. pulse 80, Bp. 104, Hb. 11.5, urea 21, weight 11.4 kgm. Animal drank total of 760 cc. water since 9:30 a.m. previous day. Urinated total 415 cc. for the 24 hours. Experiment discontinued. Dog seemed normal and was active and vigorous.

With regard to this representative protocol and graph, it is important to note that following massive injections (a total of 103 cc. given over the 56 hour period of food and water deprivation) of highly potent hormone assaying around 100 dog units per cubic centimeter, although rapid improvement followed, and the blood pressure rose simultaneously with a fall in hemoglobin, the arterial pressure, hemoglobin and urea nitrogen did not at any time attain a normal level until after fluid was taken by mouth. When the dog was permitted to drink water, the blood pressure rise and hemoglobin and urea nitrogen fall were rapid. Figure 1, protocol 1. The sharp rise in arterial pressure (29 mm. Hg in 16 hrs.) and decline in hemoglobin following extract injection were invariable responses in the animals. The new levels of blood pressure, hemoglobin and urea were not maintained but tended to slowly change toward their pre-injection levels as the period of water deprivation lengthened (fig. 1). This retrogression is associated with, and due to the loss of fluid from the dogs owing to the increased outpouring of urine following hormone injection.

Although the dog may have been anuric or practically so during the 12 to 24 hours previous to the first extract injection, the urine volume is greatly augmented after the animal has received several such injections (table 1). This loss of fluid since it is not compensated for by an equivalent intake, further dehydrates the dog and is reflected in the changes in arterial pressure and hemoglobin (fig. 1). Control experiments show that the quantity of fluid per se represented by the extract injected, is without effect upon either the blood pressure, hemoglobin or urea nitrogen.

Attention should be called to the fact that the increase in urine volume which follows extract administration to the adrenalectomized dog exhibit-

ing symptoms, and deprived of all food and water, seems to be further evidence of the mobilizing effect of the hormone upon the fluid reserves of the body.

The blood glucose was followed in several animals during the cycle of insufficiency and recovery on extract treatment. The essential data

TABLE 1

Urine volume of three dogs during the period of food and water deprivation but while receiving large amounts of cortical hormone

DOG	PERIOD OF FOOD AND WATER DEPRIVATION	EXTRACT INJECTED	URINE VOLUME	REMARKS
	hours	*cc.*	*cc.*	
48	27	62	240	No food taken during the 24 hours previous to 1st injection. Animal anuric during the interval
122	47+	80+	380	No food and only 60 cc. water during the 12 hours previous to 1st injection. Dog anuric during this interval
130	56+	103	465	No food or water taken during 12 hours previous to first injection. Animal anuric during this interval

TABLE 2

*Blood glucose changes in four typical experiments**

DOG	GLUCOSE IN MGM. PER CENT ON THE DAY EXTRACT WAS WITHHELD		MARKED SYMPTOMS PRESENT		RECOVERY, NO SYMPTOMS	
	Bp.**	Glucose	Bp.	Glucose	Bp.	Glucose
88	100		46	83	96	One glucose determination made (in duplicate) at height of insufficiency
104	105	76	50	81	100	85
106	98	84	53	85	96	82
127	104	94	50	83	102	81

* Glucose determinations were made on tungstic acid filtrate with the Somogyi modification of the Shaffer-Hartman reagent.

** Bp. = mean arterial pressure in mm. Hg.

are recorded in table 2. Significant alterations of the blood glucose from the level usual for the adrenalectomized dog in good health were not observed, even when the animals exhibited severe symptoms with marked lowering of the arterial pressure and increased hemo-concentration. It is apparent that hypoglycemia is not an important factor in these experiments.

GENERAL CONSIDERATIONS. The data presented in figure 1 and protocol 1, representative of the eight experimental animals used, indicates that the adrenalectomized dog exhibiting severe symptoms with diminished volume of circulating blood, low arterial pressure and marked hemoconcentration, does have sufficient fluid (and salt) in the tissues and interstitial spaces to bring about revival of the animal to the point where to all external appearances, and in so far as activity, appetite and vigor are concerned, the animal appears and acts normal. The first essential point in the syndrome of adrenal insufficiency is simply that the adrenalectomized animal in the absence of the cortical hormone is unable to mobilize the fluids (and salt) of its tissues for use in maintaining a normal volume of circulating fluid. When once these substances are mobilized (the mechanism of mobilization by the cortical hormone remains obscure) they are rapidly transferred to the blood stream to bolster up a failing circulation. Conditions in the adrenalectomized dog appear to be extremely favorable for blood dilution (Swingle, Pfiffner, Vars, and Parkins, 1934) providing the animal could mobilize his body fluids. This, however, the animal is unable to do in the absence of the cortical hormone.

Attention has been called to the fact that the adrenalectomized dog presenting severe symptoms and verging on prostration does not have sufficient reserve of fluid within the tissues and interstitial spaces to completely restore to normal the lowered volume of circulating blood, despite repeated administration of excessive doses of cortical hormone. The dogs have enough fluid reserves, when mobilized, to carry them well along the road to recovery, but the quantity falls short of the amount requisite for attainment of full and complete normality, as shown by the blood pressure, hemoglobin, and blood urea nitrogen levels. Figure 1, protocol 1. The second essential point in adrenal insufficiency is lack of sufficient fluid for the maintenance of a normal circulation.

Several investigators have recently shown that there is no appreciable reduction of the water content of the liver, muscle, skin and skeletal tissue of the adrenalectomized rat, cat and dog dying of insufficiency. (Hartman et al., 1932 rats; Silvette and Britton, 1933 rats and cats; Harrop et al., 1933 dogs and rats.)

The body fluids of the adrenalectomized dog off extract are lost slowly over a period of days by way of the urine, feces and expired air. When symptoms develop, the animal refuses food and the water intake greatly diminishes. Loeb et al. (1933) report that the adrenalectomized dog during the period of relatively good health and before symptoms appear, excretes by way of the kidneys much greater quantities of fluid than the same animal does over the same time interval before adrenal extirpation. The data as given by these workers are difficult to interpret because no figures are given for either the total or daily food and water intake of the animals. Without such data the urine volumes alone mean little.

Water balance studies made in this laboratory (to be published) do show, however, *that in relation to the total fluid intake* (including that of the food), the adrenalectomized dog off extract puts out more urine than while receiving hormone. The normal ratio of fluid output to fluid intake is unbalanced. The animal maintains a fairly normal daily urine volume in face of a decreased water and food intake. The daily difference in the intake and output ratio is small, but nevertheless when taken over a period of days, becomes considerable.

The tissues and interstitial spaces of the adrenalectomized dog prostrate from insufficiency, are by no means entirely depleted of their stores of fluid. (See references to Hartman et al., Silvette and Britton and Harrop et al.) If such were the case it would be exceedingly difficult to account for the sharp rise in blood pressure, and fall in hemoglobin, which follow injection of cortical extract, in the absence of fluid or food intake. For example, one of this series of experimental dogs was without food and water from the time of extract withdrawal until verging on prostration from insufficiency—a period of five days. The arterial pressure rose 23 mm. of Hg within 4 hours and 30 minutes following extract administration and in the total absence of any food or water intake during this five day interval. No further rise could be obtained despite repeated injections until the animal was permitted to drink water.

To date, our studies on blood pressure and hemoconcentration in the adrenalectomized dog tend to the same conclusion stated in an earlier paper (Swingle, Pfiffner, Vars, Bott and Parkins, 1933). The effect of the cortical hormone upon blood pressure and hemoconcentration appears to be indirect, it raises the pressure and decreases the hemoconcentration by increasing the plasma volume, i.e., the amount of fluid (and salt, Loeb et al., 1933; Harrop and Weinstein et al., 1933) in circulation, and in no other way so far as we have been able to determine. It is possible that in the dog prostrate from insufficiency the generalized stimulating effect of the mobilized electrolytes upon the efficiency of muscle tissue, and particularly the musculature of the circulatory organs, may be a factor in the rise of arterial pressure following extract administration. However, since in the adrenalectomized dog the cortical hormone mobilizes both fluid and salt simultaneously, we have not been able to devise means (with the equipment at our disposal) to separate blood volume effect from a generalized electrolyte stimulation, if the latter exists.

SUMMARY AND CONCLUSION

1. Eight animals were used in the experiments with similar results in all cases.

2. Adrenalectomized dogs, exhibiting severe symptoms and deprived of food and water for periods varying from 24 to 72 hours while receiving

extract, are capable of mobilizing sufficient fluid (and salt presumably) in the tissues and interstitial spaces for transfer to the blood stream to permit revival of the animal to the point where to all appearances, and in so far as activity, appetite and vigor are concerned the dog appears and acts normal. The blood pressure, hemoglobin and urea nitrogen do not however attain their normal levels.

3. Animals presenting severe symptoms and verging on prostration can be restored to apparently normal health by injection of cortical hormone without food (or salt) intake, but some fluid is required for complete restoration of the blood pressure, hemoglobin and urea nitrogen to normal levels.

4. The adrenalectomized dog in the absence of the cortical hormone is apparently unable to mobilize the fluid of its tissues and interstitial tissue spaces for use in maintaining a normal volume of circulating fluid.

5. Hypoglycemia cannot be regarded as an important factor in these experiments, since significant alterations of the blood glucose were not observed in this series of experimental animals, even when they exhibited severe symptoms.

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A STUDY OF THE PROPULSIVE ACTIVITY OF A THIRY-VELLA LOOP OF INTESTINE[1]

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The motor activity of the gastro-intestinal tract may be divided into movements which transport material and those which mix contents without appreciably altering its location. Factors which modify motility of the gut may not influence this propulsive and non-propulsive motility in a similar manner. Recognition of these facts is generally imperative for complete appreciation of gastro-intestinal motor phenomena. Failure to consider these distinctions constitutes a serious interpretive defect in numerous physiological, pharmacological and pathological investigations of intestinal motility.

The balloon method, as it is usually employed, does not permit a satisfactory distinction between propulsive and non-propulsive motility. The fluoroscopic method is more satisfactory from this standpoint but there are numerous investigations to which it is unsuited and others in which it does not yield the detailed information desired. The methods by which propulsion of liquids in isolated strips of the intestine have been studied were reviewed by Sollmann and Rademakers (1) and in addition they have used their modification of this principle for studies of propulsion rate. Unanesthetized dogs were employed in the investigation of Schüpbach (2); a lump of sealing wax was introduced into the oral end of a Thiry-Vella loop of the intestine and propulsive rate determined by noting the rapidity of disappearance of a piece of string attached to the bolus. Cash (quoted by Pavlov (3) p. 195) passed the thread from the bolus over a pulley and studied the movement of a weight hung from the thread. The time required for a bolus of cotton and gauze to pass from the oral to the aboral end of a Thiry-Vella loop was investigated by Reid (4).

The principle employed by Reid appeared to possess the greatest number of fundamental advantages and we have therefore elected to employ a modification of this method for a detailed study of intestinal propulsive motility and the factors which modify it in the unanesthetized dog.

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Operative procedure. This investigation quickly demonstrated that certain details in the operative technique were essential for the preparation of a Thiry-Vella loop which would yield consistent results. In brief, these details are as follows: *a*, the portion of the loop within the abdominal cavity of an average sized dog (10 to 15 kgm.) should not exceed 9 to 12 cm. in length; *b*, the ends of the loop should be secured to the abdominal wall with a tension sufficient to prevent the formation of pockets or kinks in the loop; *c*, the loop should be of a length which allows the projection of each end 2 to 3 cm. beyond the skin after the sutures are in place; *d*, the stab wounds in the abdominal wall should be sufficiently large and the sutures so placed that even after healing is complete the lumen of the loop is not constricted in its passage through the abdominal wall. The entire procedure is learned after some experience. Two or more loops may be prepared in the same animal and they may be placed either longitudinally or transversely to the body axis.

Choice of bolus. We have investigated or considered a number of substances which could be introduced within an intestinal loop for a determination of propulsive motility. Tight fitting cannulae for the introduction and collection of solutions (adaptation of the Sollmann-Rademakers technique to the Thiry-Vella loop) were made of rubber nipples. Although they were held in place only by the elasticity of the rubber they retarded venous return sufficiently to change the color of the loop ends; furthermore, well-trained animals became restless and the propulsion rate was erratic.

The use of solutions in the intestinal lumen of unanesthetized dogs raised the confusing possibility of the fluid passing through the intestine in the absence of true peristaltic activity. This could be the result of a localized pump-like movement in one portion of the loop, or some unknown factor for Alvarez ((4), p. 214) has noted the passage of fluids for long distances in the exposed gut even when peristalsis could not be detected. Errors related to intestinal secretion or absorption may also occur when propulsion is studied by measuring the rate at which a fluid passes through the intestinal loop. We attempted to avoid such objections by using an indifferent liquid, mineral oil, but again the technical difficulties related to the introduction and recovery of liquids for quantitative measurements appeared to overbalance the desirable features of this method.

From theoretical considerations it was decided that an inert, insoluble and readily yielding bolus of a size, shape, consistency and specific gravity approximating normal intestinal contents would be most suitable. It was found that the cotton and gauze bolus of Reid was too rough and unyielding for our purposes. Of the various substances tested, the bolus which approximated the theoretical restriction imposed and also gave the most uniform results was obtained by cutting a small cylinder from a piece

of fine grade crepe ("sponge") rubber. The cylinder was pointed at both ends and grooved in the middle to increase its flexibility. After trying boli of various sizes in a number of dogs of average size, it was concluded that a bolus measuring 2.5 × 0.8 cm. is most universally satisfactory. (We do not deem it necessary to give the data obtained in this portion of the investigation which shows that a large unyielding, rough bolus passes through the loop at a less uniform and slower rate.) Such crepe rubber boli were soaked over night in liquid petrolatum to increase their smoothness and pliability. With these boli many control determinations of propulsion rate may be obtained in a relatively short period of time.

Method employed in determining propulsive rate. When the interval required for the passage of a rubber bolus through a Thiry-Vella loop was repeatedly determined, propulsion time was observed to be initially very long and to gradually shorten as repeated trials were made. For example, in one experiment the first five times the bolus was inserted into the loop the propulsion times respectively were: 14, 12, 11, 8, 8 minutes. The initial slow propulsion was apparently related to a deficiency of intestinal lubrication for it was almost completely eliminated by the routine introduction of 2 cc. of liquid petrolatum into the loop each day at the beginning of the experimental period. This procedure would also minimize the effect on bolus propulsion rate which variations in the quantity and quality of intestinal secretion might otherwise produce.

The routine finally adopted consisted of the introduction of oil, the bolus was carefully inserted with blunt forceps into the oral end of the loop and the time noted which was required for its reappearance at the aboral end. The bolus was immediately reinserted and the procedure repeated until several uniform results were obtained. Any factor to be studied was then introduced and its effect investigated by continuing the determinations of the bolus rate.

If pocketing of the bolus occurred during an experiment, it would, of course, lead to erroneous results. To guard against this contingency we made it a rule to remove the bolus whenever an unexpectedly long propulsion time appeared and make a redetermination. A soft rubber catheter was used for this purpose. It is also interesting to note that the insertion of a second bolus would force out the pocketed bolus. If pocketing in a certain loop occurred frequently the animal was discarded.

Normal propulsion rate. When attention was given to the details considered in this paper, a striking degree of uniformity in normal propulsion time was usually observed over periods of several hours (some of our experiments continued for eight hours). For example, propulsion rate determined repeatedly during a two hour interval did not vary by more than one minute from the median. At rare intervals an abnormal propulsion interval might develop but this could usually be ascribed to one

of the factors considered in this paper (vide infra) which modify propulsive activity and evaluated accordingly.

The normal propulsion rate of an intestinal loop remained rather constant from day to day for periods as long as three months, the usual range in variation of median rates during such a period being within three minutes. It was noted, however, on several occasions that propulsion time was as much as double the normal. The propulsion time on such days was also found to be rather erratic; successive determinations might vary by as much as 100 per cent. It was usually possible to relate such abnormal results to a psychic disturbance of the animal, gastro-intestinal upset associated with loose bowel movement, etc., but such unusual results were also encountered on occasions when a satisfactory explanation was not available. If the abnormal results were related to the animal being restless, normal propulsion could generally be regained by calming the animal. The Thiry-Vella loops from the jejunum in each of the five dogs used extensively in this investigation measured 9 cm. between the two points of exit from the abdominal wall. The normal propulsion time of three of these dogs was 6 minutes, in the two animals remaining it was 5 and 7 minutes respectively. Taking propulsion rate as 6 minutes for 9 cm. (1.5 cm. per minute) and the normal length of the small intestine of a 10 kgm. dog as 300 to 400 cm. (Ellenberger and Baum (6) p. 296; Flint (7)), the time required for passage of a bolus through the entire small intestine was calculated to be 200 to 266 minutes. This calculation, of course, fails to consider the type of propulsive activity in different portions of the intestine (e.g., the duodenum and lower ileum) which would be expected to vary considerably (5), (8). In a limited number of studies, however, the average propulsion rate was found to be only slightly slower (8 to 9 minutes for 9 cm.) in loops made from the lower ileum.

The normal propulsion rate in the experiments of Schüpbach was very slow, apparently due to the failure of the investigator to observe the essential precautions noted in this paper. His bolus was propelled 4 cm. in 34 minutes in one experiment and 9 cm. in 23 minutes on another trial. A bolus introduced into the intestine exposed under saline was propelled at the rate of 0.5 to 2 cm. per minute (Pavlov (3) p. 195) and required 2 to 3 hours to travel the whole length of the intestine. Krzywanek (9) obtained a range of 75 to 180 minutes as the elapsed time between the beginning of emptying of a barium meal into the intestine of a dog and its first appearance in the colon. While it is of interest to compare the propulsion rate of a barium meal and the rubber bolus it should be recalled that, in general, the more fluid the material the more rapidly it passes through a given portion of the gastro-intestinal tract. The contrast between the passage of fluids and solids through the esophagus and the passage of barium suspension and the rubber bolus through the intestine is

not identical but in regard to the point under consideration they do have a general similarity.

The influence of emotions on intestinal propulsion. Studies made by the balloon or fluoroscopic method have generally indicated that emotions depress the motility of the stomach and intestine (Pavlov (3); Cannon (10); Carlson (11)). However, Barcroft and Robinson (12) did not observe a change in motility of an exteriorized intestinal loop during emotional states but Borchardt (13) found an increase of intestinal tone and motility related to pleasant emotions and decreased motor activity when the emotions were unpleasant.

We found that progress of the bolus through the intestinal loop was usually but not invariably retarded when the animal became restless. In an experiment typical of those in which delayed propulsion occurred, the successive determinations of normal propulsion times expressed in minutes were: 6, 7, 6, 6, 6. The slamming of a door then resulted in a prolonged period of trembling during which the animal raised its head several times as if an attempt to stand were to be made. During this interval propulsion times were 5, 8, 11, 7, 6, 6. While an animal was in the process of being trained, the time required for the passage of the bolus was long and irregular. More rapid and uniform propulsion developed coincidently with the well trained condition and the suppression of emotional states. We were unable to relate variations in intestinal activity to certain types of emotions as was done by Borchardt but in order to obtain uniform results we minimized their importance by employing well trained animals, i.e., dogs which would not lie quietly and fully relaxed even in the presence of mildly disturbing factors were discarded.

The effect of ingestion of a meal on propulsive activity. The upper intestine as shown by balloon studies, manifests motor activity during fasting which is closely related to gastric hunger contractions (Ivy and Vloedman (14); Sinelnikov (15); Quigley and Solomon (16)). This relationship does not exist in a portion of the intestine which has lost its anatomical continuity with the remainder of the gut as in the Thiry fistula and the motor activity of such an intestinal preparation is not affected by the entrance of food into the stomach (14). In the experiments of Cash (quoted by Pavlov (3) p. 195) a bolus moved through the intact intestine of a laparotomized animal during fasting at the rate of 0.1 to 0.5 cm. per minute but after feeding the speed was 1.5 to 2 cm. per minute. When food was ingested the movements of an exteriorized intact loop of the intestine were increased (Barcroft and Robinson (12)).

In a large number of experiments in which we determined propulsive time by the bolus method, the rate was found to be uniform and to be within the same range (1.8 to 1.5 cm. per minute) whether the determinations were made 10 minutes or 24 hours after feeding. The apparent

contradiction between our results and those of Cash or of Barcroft and Robinson may be related to their use of the intact intestine while we employed an isolated (Thiry-Vella) loop.

The effect of disuse of the Thiry-Vella loop on propulsive motility. It has generally been observed that when a portion of the gut is separated from the remainder of the tract (as in gastric pouches or the Thiry-Vella loop) so that it no longer serves its normal function, certain motor and secretory changes in the tissue occur. These phenomena are prevented by frequent stimulation (especially mechanical) of the tissue (Cash (17); Savish (18); Babkin and Sinelnikov (19)) and are referred to as disuse phenomena.

We observed with each of our dogs at the beginning of the bolus experimentation (7 to 9 days post-operatively) a slow and irregular propulsion rate (0.2 to 0.3 cm. per minute). A similar condition was noted by Barcroft and Robinson but Babkin and Sinelnikov reported increased motility which persisted for 2 or 3 weeks. Since our animals at this time were untrained, restlessness may have been a factor; however, a delay in intestinal propulsion at least as extensive was also seen in well trained animals in which bolus experiments were again undertaken after a respite of several months. The condition is typically illustrated by an experiment performed on a dog which had a normal propulsion time of 4 to 6 minutes during a series of experiments which continued for three months. Subsequently the animal was not employed for bolus experiments until two and one-half months later. The first series of determinations made at this time were: 27, 32, 30, 26, 21, 18, 20, 16, 12, 12, 14, 10, 10, 8, 7 minutes. On the following day the results were: 34, 20, 16, 12, 8, 6 minutes.

The marked retardation of intestinal propulsion during the period of disuse and the counter effects of using the loop may be related to an alteration in intestinal secretion. The secretion of a Thiry-Vella loop which is not being used becomes solidified and wax-like and would have less lubricating action than normal secretion. However, delayed propulsion persists in a thoroughly lubricated loop, in fact the loop from which the results detailed above were obtained was lubricated by the introduction of 3 cc. of mineral oil preceding the experiment. Alterations in the anatomical condition of the loop such as occur in disuse of skeletal muscle do not appear to be responsible for the slow propulsion. This is exemplified by the return of normal propulsion rate in the experiments detailed in the preceding paragraph as the result of repeated introduction of the bolus for 4 hours and 30 minutes on the first day and only 90 minutes the following day. Furthermore, we have confirmed the observation of Reid (4) that normal propulsive activity may be restored to a loop showing the disuse phenomena by the intravenous injection of hypertonic sodium chloride solution. An increase in intestinal tone (to the extent that introduction of the bolus of normal size becomes difficult) which dominates

all motor activity of the loop is probably the prime factor involved. The pale appearance and firm feel of the unused loop should be recalled in this connection. A depression of peristaltic movements is probably also involved in the production of the slow propulsion. Development of the alteration in propulsive motility related to disuse may be prevented by frequent employment of the intestinal loop. Bolus experiments should be performed daily if uniform propulsion is desired.

It is well known that a Thiry-Vella fistula which has not been used for several weeks will give balloon records which are generally accepted as indicating normal intestinal motility. Our observations show that this motility is not normal, but is almost exclusively of the non-propulsive type and is associated with abnormally high intestinal tone. The desirability of distinguishing propulsive from non-propulsive motility is thus further emphasized.

Discussion. The bolus method distinguishes the propulsive type of intestinal motility from the non-propulsive type, but propulsion rate is still the resultant of several factors. It is modified especially by the tone of the longitudinal and circular muscle fibers, the frequency and amplitude of the peristaltic waves, etc. A marked increase in postural tone of the loop either local or general might result in stagnation of the bolus in spite of moderately vigorous peristalsis. There may be a disturbance of the reciprocal relation between the longitudinal and circular muscle which relation is necessary for peristaltic efficiency (Sollmann and Rademakers (4)). It should be emphasized that the bolus method only measures the rate at which the bolus is transferred from one portion of the gut to another. That is, it gives information regarding the rate at which the particular type of intestinal contents is propelled under the condition existing. It does not tell the rate at which every other type of material would be propelled (e.g., the propulsive rate of mineral oil is 2 to 3 times that of the bolus). However, it appears not to be an unwarranted assumption that the propulsion of other contents is directly related to bolus rate, conditions altering one will alter the other in the same direction and to the same extent.

The bolus method is well adapted to the study of the action of a great variety of physiological, pharmacological and pathological factors on intestinal propulsive motility. The same animal may be used for an infinite number of determinations. The results of different procedures may be compared on the same or different animals. If two or more loops are employed in one dog, propulsion may be simultaneously compared in several portions of the gut (e.g., duodenum, jejunum, ileum and colon). The method may be modified slightly to permit its employment on the intact intestine. The bolus method appears to give indisputable evidence regarding propulsive activity; nevertheless, caution must be exercised in

transposing the conclusions from bolus studies in the dog to intestinal propulsion in man. This is particularly true when the so called normal intestine of the Thiry-Vella loop is compared with the abnormal intestine in paralytic ileus. Bolus studies may be used as a starting point for studies on propulsive motility in experimental ileus and a study of the remedies used for ileus and their limitations.

SUMMARY

Intestinal propulsive activity has been accurately determined by observing under suitable conditions the rate at which a bolus was propelled through an intestinal loop. When conditions were unchanged the propulsion rate as repeatedly determined in jejunal Thiry-Vella loops of well trained, unanesthetized dogs was practically constant (1.5 cm. per minute). This rate persisted for months; it was not modified by feeding but usually became slower and erratic if restlessness supervened. Disuse of the intestine led to marked prolongation of propulsion time, but this was readily overcome by use of the intestine or the intravenous injection of hypertonic saline solution. The propulsion rate in loops of the lower ileum was slightly slower than in the upper jejunum.

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THE EFFECT OF SODIUM CHLORIDE ADMINISTRATION UPON ADRENALECTOMIZED DOGS NOT RECEIVING EXTRACT[1,2]

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Rogoff and Stewart (1925, 1928) called attention to the striking effect of intravenous injections of Ringer-dextrose solution upon the syndrome of adrenal insufficiency, and demonstrated decisively that daily injections (about 100 cc. per kilogram of body weight) markedly prolonged the life-span of the operated animals. They explained the beneficial action of the injections by assuming "the possibility that a poison or poisons accumulating in the absence of the adrenals may be washed out of the tissues (or perhaps neutralized) by the thorough irrigation caused by the large injection of Ringer's solution."

Banting and Gairns (1926) confirmed these findings on dogs in so far as amelioration of symptoms is concerned. Marine and Baumann (1927) and Corey (1926–27) using cats and administering the saline by mouth obtained somewhat similar results. Baumann and Kurland (1926–27) called attention to the fact that the plasma sodium and chloride content of the blood decreases in the bilaterally adrenalectomized animal exhibiting symptoms.

Loeb (1932) gave impetus to renewed investigation of the problem by demonstrating a lowering of the sodium content of the blood serum of a patient with Addison's disease.

Recently, Loeb et al. (1933b) found that in dogs the sodium concentration of the blood decreased with the loss of the adrenals. The sodium was lost from the body by way of the urine during the development of insufficiency. It was suggested that possibly the adrenal glands exert a regulatory effect upon sodium metabolism.

These observations have been confirmed by Harrop (1933b, c, d) and

[1] In the collaborative studies on the adrenal cortex carried on in this laboratory the writers have specialized in certain phases of the experiments. The physiological aspect of the problem is the responsibility of the senior author (W. W. S.); the biochemical work of extraction and purification of the hormone that of one of us (J. J. P.).

[2] The expenses of this investigation have been aided by a grant from the Josiah Macy Jr. Foundation, New York.

collaborators on adrenalectomized dogs, and patients with Addison's disease.[3]

The present work was undertaken to determine whether or not administration of sodium chloride in large quantities to bilaterally adrenalectomized dogs could in any way substitute for the cortical hormone, and keep the animals in normal condition after extract was withdrawn.

EXPERIMENTAL. Bilaterally adrenalectomized dogs were used; they had well healed wounds, were normal[4] animals at or near their peak weight.

TABLE 1

Effect of feeding large quantities of sodium chloride on the survival period of adrenalectomized dogs following extract withdrawal

DOG	SEX	WGT.	OPERATIVE AGE	NaCl	FOOD OFFERED	DURATION OF EXPERIMENT	REMARKS
		kgm.	*days**	*gms./kgm./day*	*grams*	*days†*	
88	♂	12.0	31	2	600 K.R.**	19	Severe symptoms
99	♂	10.0	48	2	500 K.R.	55	Severe symptoms.
104	♂	9.6	14	3 for 5 days 2 for 8 days	600 K.R.	14	Severe symptoms
107	♀	14.2	29	2	700 K.R.	21	Severe symptoms
114	♂	13.6	39	2 for 2 days 1 for 11 days	700 K.R.	14	Severe symptoms
130	♂	12.5	20	1	400 B‡	12	Severe symptoms
143	♀	11.3	20	1	700 K.R.	14	Severe symptoms

* Operative age—Interval between bilateral adrenalectomy and beginning of salt feeding experiment.

** K.R.—Ken-L-Ration.

‡ B—fresh ground beef.

† Interval between extract injections figured from time of last injection previous to feeding salt and injection when the animal was exhibiting moderate to severe symptoms of insufficiency.

From the day of gland removal they were injected daily with adequate doses of cortical hormone until ready for use. In this set of experiments,

[3] After this paper was written, a preliminary paper by M. I. Rubin and E. T. Krick came to our attention. These investigators state that by using $CaCl_2$, MgCl, NaCl and KCl they were able to revive comatose rats and to keep them in apparent health for four months on this mixture. It was their impression that 0.9 per cent NaCl was less effective when used alone than when other salts were added.

M. I. Rubin and E. T. Krick. 1933, Proc. Soc. Exp. Biol. and Med., **31**, 228.

[4] The Hb. of bilaterally adrenalectomized dogs is generally, if not invariably considerably lower than normal. The experimental details relating to this observed anemia will be presented elsewhere. Suffice it to state here, that our data indicate that the lowered Hb. is due to loss of the adrenals, and not to dietary or other disturbing factors.

the period intervening between complete adrenalectomy and experimental use, varied from 14 to 48 days (table 1).

The diet of the dogs during the actual period of experimentation consisted of Ken-L-Ration (Chappel Bros., Rockford, Illinois) and sodium chloride. The usual yeast, cod liver oil, and mineral salts which it is the custom in this laboratory to add to the basal meat diet of our dogs were omitted. Occasionally, when the animals refused the Ken-L-Ration, fresh finely ground beef plus the salt was given for a day or two.

Reference to various methods employed for determination of blood pressure, hemoglobin (abbreviated to Bp. and Hb. in the protocols), blood

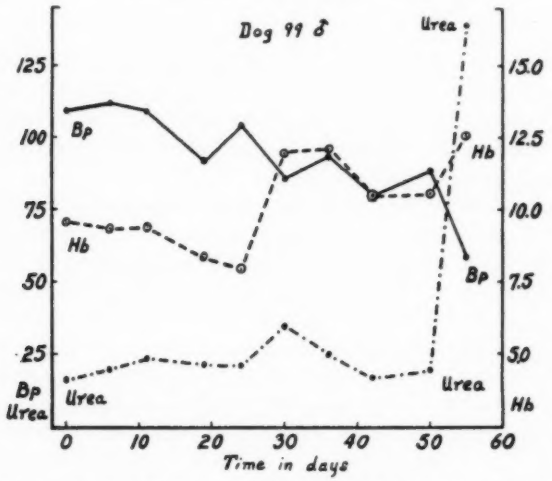

Fig. 1. Blood pressure (Bp. in mm. Hg), hemoglobin (Hb. in grams per cent) and blood urea nitrogen changes (in mgm. per 100 cc.) in an adrenalectomized dog (no. 99) receiving daily 2 grams of NaCl per kilogram of body weight. This animal had been bilaterally adrenalectomized 48 days previous to the beginning of the experiment. The salt feeding was discontinued on the 50th day.

urea nitrogen, and glucose, are as follows: blood pressure in mm. of Hg. Dameshek and Loman (1932); Parkins (1934); urea mgm. per cent, Van Slyke manometric hypobromite method (1929); hemoglobin, grams per cent, Newcomer; glucose, mgm. per cent Somogyi (1926) modification of the Shaffer-Hartman reagent. A summary of the data is given in table 1. One protocol typical of the animals used in the salt feeding experiments is given below. Figure 1 gives the data on one long surviving animal.

Protocol 1. Dog 114. Adult male. Bilaterally adrenalectomized 3/9/33. Maintained in normal health by adequate doses of cortical extract until 4/18/33. Weight

13.6 kgm. Bp. 108, pulse 80, Hb. 10.7, urea 15, glucose 82. Dog given 2 grams of NaCl per kgm. of body weight daily in 700 grams of Ken-L-Ration with free access to water. The dog refused to eat all of the heavily salted food. On 4/21/33 salt reduced to 1 gram per kgm. and the dog ate his ration. No more difficulty with the food was encountered. On 4/24/33 Bp. 100, pulse 80, Hb. 12.0, urea 28, glucose 83. On 5/1/33 at 10:30 a.m. Bp. 70, pulse 72, Hb. 16.2, urea 23, glucose 81. The animal seemed weak and somewhat spastic. He refused all food and was given 2.8 grams of NaCl in 2 capsules. At 11 a.m. he vomited the salt and failed with unusual rapidity. At 2:50 p.m. dog was barely able to walk and had vomited violently several times since morning. Bp. 42, pulse 140, Hb. 18.4, urea 52, glucose 65. The experiment was discontinued and the dog returned to normal with cortical extract and the usual diet. This protocol is typical of the group of salt fed animals. The daily urine output and water intake were greatly increased during the period of salt feeding.

Intraperitoneal injections of saline. Since the efficacy of daily intravenous injections of Ringer-dextrose solution upon adrenalectomized dogs had been so clearly demonstrated by Rogoff and Stewart we made no effort to experiment along these lines. However, a few tests of the effect of intraperitoneal injections were made.

Intraperitoneal injections of saline appear not to be effective when given to dogs exhibiting very severe symptoms of insufficiency; such as arterial pressure at the shock level, marked hemoconcentration and high blood urea nitrogen. Animals presenting such symptoms seem to be unable to utilize to advantage the salt and water injected and succumb unless the cortical hormone is injected.

On the other hand, dogs which exhibit mild symptoms and are still able to be up and around, respond to repeated injections of large quantities of saline and remain in apparently normal physical condition for considerable periods. The urea, hemoglobin and blood pressure of such animals return to their normal levels. We have performed four such experiments; the results appear sufficiently clear-cut to demonstrate the points involved.

A. *Severe case. Protocol 3.* Dog 75. Adult male. Weight 12.3 kgm. Bilaterally adrenalectomized 1/1/33. Maintained in normal health by adequate doses of cortical hormone. 2/8/33 last injection given. Pulse 92, Bp. 99, Hb. 9.1, urea 23, glucose 87. On 2/15/33 at 11:20 a.m. animal exhibited symptoms of insufficiency, was weak, spastic and swayed when walking. Pulse 92, Bp. 51, Hb. 11.5, urea 65, glucose 84. Animal vomited bile stained fluid at 11:20 a.m. At 11:40 the dog was injected intraperitoneally with 800 cc. of 0.9% saline. 2 p.m. pulse 88, Bp. 46. At 2:30 p.m. pulse 84, Bp. 43, Hb. 11.3, urea 58, glucose 89. The animal was failing rapidly and was in collapse. Given intravenous injection of 36 cc. of cortical extract. At 2:45 p.m. pulse 76, Bp. 38. Animal unable to rise to feet. At 7 p.m. pulse 152, Bp. 61. Dog was up walking about. The improvement was astonishing, considering that at 5:30 p.m. the animal could not get on his feet. At 11 p.m. pulse 160, Bp. 66, Hb. 10.3, urea 75, glucose 72. Left in cage overnight without food or water. 2/16/33 at 9:30 a.m. pulse 152, Bp. 70, Hb. 9.8, urea 49, glucose 81. Dog given 20 cc. extract intraperitoneally, 500 cc. milk, 500 cc. water and 500 grams Ken-L-Ration placed in cage. At 2:15 p.m. pulse 144, Bp. 80. Dog had taken 500 cc. milk, plus 399 cc.

water since 9:30. Animal given 15 cc. extract intraperitoneally 4:45 p.m. At 7:20 p.m. pulse 140, Bp. 95, Hb. 9.0. Dog seemed perfectly normal. Had taken additional 150 cc. milk since 2:15 p.m. Following morning 2/17/33 at 10:10 a.m. pulse 124, Bp. 100, Hb. 9.4, urea 23, glucose 80. Dog had taken 270 cc. milk plus 30 cc. water since previous evening. Animal normal. Experiment discontinued. Figure 2 is a graphic representation of the arterial pressure and hemoglobin changes in this animal throughout the experiment.

B. *Mild case.* The essential data concerning the type of experiment where the adrenalectomized dog presenting mild symptoms of insufficiency

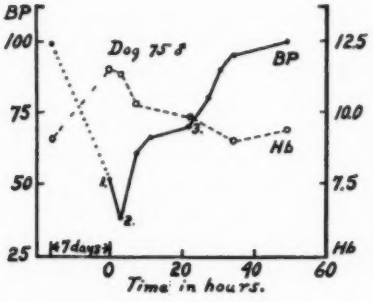

Fig. 2

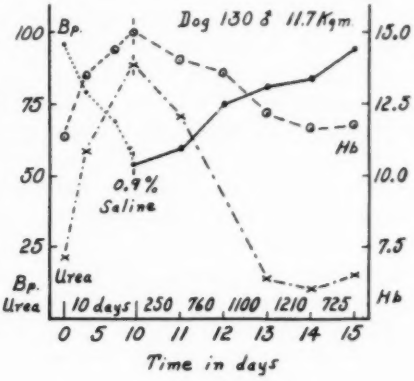

Fig. 3

Fig. 2. Blood pressure and hemoglobin changes in an adrenalectomized dog (no. 75) exhibiting severe symptoms of insufficiency. The adrenals had been removed 38 days previous to beginning of the experiment. Cortical extract was withheld for 7 days. The Bp. and Hb. changes during the 7 day interval are not plotted in the graph, only the initial levels of Bp. and Hb. are indicated. *1* = 800 cc. of 0.85 per cent saline injected intraperitoneally; *2* = intravenous injection cortical extract but all fluids withheld; *3* = animal permitted to drink water.

Fig. 3. Blood pressure (Bp. in mm. of Hg), hemoglobin (Hb. in grams per cent) and blood urea nitrogen (mgm. per 100 cc. of blood) changes in an adrenalectomized dog (no. 130) not receiving extract and exhibiting symptoms while receiving daily 1000 cc. of 0.9 per cent saline intraperitoneally. Ten days elapsed from date of extract withdrawal to the time of first saline injection. This dog had been bilaterally adrenalectomized three months previously and maintained in normal health by injections of cortical extract. The figures just above the base line and beginning with the day on which saline was injected, represent the urine volume in cubic centimeters.

was intraperitoneally injected with saline are given in figure 3. This animal although showing slight symptoms was up and about when injected and received 1000 cc. of 0.9 per cent sodium chloride daily for five days. The saline was given in two injections of 500 cc. each morning and evening. At the end of the injection period the animal appeared

normal in so far as activity, vigor and appetite were concerned. The Bp., Hb., urea and pulse were at their normal levels (fig. 3).

GENERAL CONSIDERATIONS. The daily ingestion of sodium chloride in large quantities mixed with the ordinary ration, prolongs somewhat the life-span of the bilaterally adrenalectomized dog. Table 1. The great majority of our adrenalectomized dogs on ordinary rations develop symptoms of insufficiency anywhere from 4 to 12 days after withdrawal of the hormone. The longest interval observed was 16 days. In view of Loeb's (1933a) report of his results with sodium chloride administration to patients with Addison's disease, we had anticipated a much longer period of normal health for the salt-fed dogs. It is quite true that the conditions existing in Addison's disease and in the bilaterally adrenalectomized dog differ considerably. The human patient generally has some remnants of adrenal cortex present (Rowntree and Snell, 1931). The adrenalectomized dog, on the other hand, is devoid of such tissue.

The dogs on the high salt diet remained in comparatively good health so long as they ate the salty food and did not suffer gastro-intestinal upsets. The observation was repeatedly made, that the salt-fed dogs were subject to gastro-intestinal upsets, and if they vomited, a crisis was precipitated which necessitated prompt measures in order to save the animal's life (protocol 1). This protocol is representative of the data obtained on all of the salt-fed dogs with the exception of dog 99. The essential data obtained on this animal are shown in figures 1 and 4. Dog 99 was maintained in fairly normal condition for 50 days on the salt diet and quickly developed insufficiency when salt was withdrawn. The blood pressure was invariably lowered in these animals, although they looked and behaved like normal dogs. Even in the dog surviving 55 days (dog 99) the blood pressure was subnormal for days before he finally refused to eat the salty food. However, despite the lowered arterial pressure the animal seemed in fair condition (fig. 1).

The results of these experiments convince us that feeding NaCl to the point of tolerance, and allowing free access to water, cannot substitute indefinitely for the cortical hormone. Such treatment is palliative for a brief period but sooner or later a crisis is precipitated usually by a gastro-intestinal upset or by the animals' refusal to eat the salty food, and recourse to the cortical hormone is necessary. Rogoff and Stewart's (1925, 1928) experiments also demonstrate this fact clearly, in so far as intravenous Ringer-dextrose therapy is concerned.

Harrop, Weinstein et al. (1933b) have recently expressed somewhat similar views with especial reference to the Addison's disease patient.

The cortical hormone is requisite for the mobilization and maintenance of the normal water and salt content of the blood and tissues. Placing fluid and salt at the disposal of an animal in insufficiency is one thing,

but the indefinite retention and utilization of these substances by the cortical hormone deficient dog is something altogether different.

The termination of several of the salt feeding experiments was complicated by hypoglycemia (fig. 4). The lowering of the blood sugar was in all cases due to violent gastro-intestinal upsets due to ingestion of the salty food. The pronounced effect of prolonged vomiting upon the bood glucose level is shown in protocol 1 and figure 4. This figure represents graphically the principal findings with regard to the blood glucose of the salt-fed animals. In several cases (dogs 104 and 114, fig. 4)

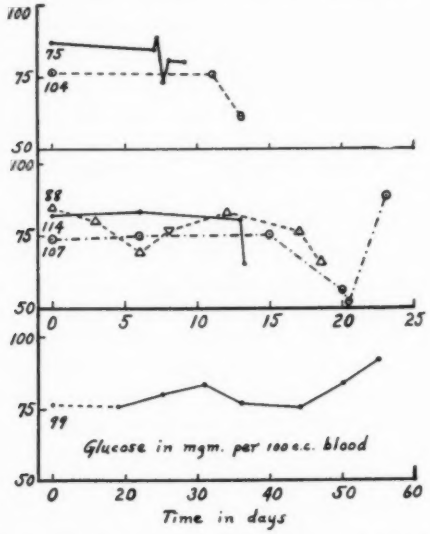

Fig. 4. Changes in the blood glucose of adrenalectomized dogs fed sodium chloride to the point of tolerance. Glucose is expressed in milligrams per 100 cc. of blood. The number of the animal is given in each case. No. 75 was injected with saline. Note that the time interval as plotted for dog 99 is different from that of the remainder of the animals.

the fall in blood glucose was a direct result of violent vomiting induced by struggling upon passage of a stomach tube, and in the other case by administration of sodium chloride in gelatin capsules. The blood glucose of one animal (dog 99, fig. 4) rose sharply during the period of insufficiency when salt had been discontinued.

In our experience covering a large number of adrenalectomized dogs on an ordinary dietary regime, hypoglycemia is exceptional. The animals exhibiting insufficiency show little change in blood sugar even when the animal is prostrate and on the verge of death, protocol 2, dog 75, figure 2.

The writers expect to take up the matter of the alleged relation of the adrenal cortical hormone to carbohydrate metabolism in a later communication. The data lead us to agree with Harrop and Weinstein (1933) that the cortical hormone is not concerned with sugar metabolism in the dog, at any rate, and the hypoglycemia occasionally encountered is probably due to a medullary deficiency.

SUMMARY AND CONCLUSION

1. Feeding sodium chloride to the point of tolerance to bilaterally adrenalectomized dogs not receiving cortical extract prolongs somewhat the interval intervening between cessation of extract injections and development of adrenal insufficiency.

2. Seven animals were used in the experiments. Three animals remained free from symptoms for 14 days, one for 12 days, one 21 days, one 19 days and one 50 days. Salt was withdrawn from the diet of the long surviving animal on the 50th day. Four days later the dog exhibited severe symptoms of insufficiency.

3. Sodium chloride and water cannot be regarded as an effective substitute for the cortical hormone in the bilaterally adrenalectomized dog since it was necessary to inject cortical extract in order to save the animals when they sooner or later developed severe symptoms.

4. A decline in the level of blood glucose is likely to develop in the adrenalectomized dog fed sodium chloride to the point of tolerance. Dogs on ordinary diets when permitted to develop severe symptoms of insufficiency by withdrawal of extract rarely show changes in the blood sugar.

5. Intraperitoneal injections of large quantities of 0.85 or 0.9 per cent saline were ineffective in relieving the symptoms of severe insufficiency. Such injections proved effective in relieving gross symptoms in two animals exhibiting mild insufficiency; the blood pressure, blood urea nitrogen, and hemoglobin were restored to their normal levels by such treatment.

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THE NEURAL MECHANISM OF MASTICATION

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Rhythmic chewing in the rabbit can be readily obtained by electrical stimulation of the cortex. This has been described by a number of investigators including Ferrier (1886), Gad (1891), Rhethi (1893), Carpenter (1895), Mann (1896), and Miller (1920). More recently Bremer (1923) has discussed the mastication in the cat and rabbit in detail. The purpose of the present investigation was first to study the localization of neural mechanisms in the cortex and mid-brain and then to analyze rhythmic chewing.

METHODS. In the *acute experiments*, rabbits and guinea pigs were used under light dial anesthesia (Fulton, Liddell and Rioch, 1930), supplemented by ether when necessary. The dose of dial was usually 0.6 cc. per kgm. given intraperitoneally.

The source of the stimulating current for purposes of localization in the cortex and mid-brain was a Harvard inductorium with 3 volts to the primary coil and the secondary coil set at 6 to 8 cm. When analyzing rhythmic chewing, rabbits were used. The stimulating current was supplied by a multivibrator similar to that described by Rosenblueth (1932) except that this machine was considerably more powerful and was run from a 110-volt alternating current line. This multivibrator was designed and constructed by Mr. E. L. Garceau, electrical engineer of the Laboratories of Physiology. Bipolar silver electrodes were used throughout. During the earlier experiments, these were applied by hand and were removed between periods of stimulation. In later experiments, and when the latent period and after-discharge of rhythmic chewing were being determined, the electrodes were clamped in place and were not moved throughout the experiment. The exposed cortex was kept warm and moist by means of saline.

The site of application of the stimulus was determined by exploring the "chewing area" and finding the point from which the response could be most readily elicited. Maximal stimuli were used except in experiments in which the intensity was the variable factor. The duration of the periods

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of stimulation was from 5 to 90 seconds. Rest periods for cortical stimulation were usually from 2 to 5 minutes; for subcortical stimulation from 30 seconds to 1 minute.

In the experiments on subcortical stimulation, the cortex was carefully removed and the regions below were then stimulated. Fixation of the animal's head was obtained by clamps in the bony meatus and a rod through the maxillary bones.

Vertical and lateral movements of the jaw were recorded separately on a smoked drum by means of levers suitably attached to a very small clamp applied to the lower incisors. The drum revolved at a rate of approximately 35 cm. per second.

In the *survival experiments* guinea pigs were used, for these animals proved to be considerably hardier than rabbits and have essentially the same type of brain. In the earlier experiments complete decortication was done in two stages. In a later series, bilateral ablations of the anterior part of the cortex, which included an area greater than that which yielded chewing on cortical stimulation, were carried out. Nembutal (sodium pentobarbital), 0.55 cc. to 0.75 cc. per kgm. intraperitoneally, was the anesthetic used, supplemented occasionally by ether. The mortality following the cortical ablations was high, due to the difficulty of feeding the animals, which in no case ate spontaneously after the cortices of both hemispheres had been operated on. Daily injections of saline and 5 per cent glucose intraperitoneally and subcutaneously were of value.

At the termination of these survival experiments the animal was anesthetized by intraperitoneal injection of dial (0.55 cc. per kgm.), supplemented by ether. The brain stem was transected at successive levels down to the pontile region and the cut surface stimulated with bipolar silver electrodes connected with the secondary coil of a Harvard inductorium. The current used was of the same intensity as that required to elicit chewing in a normal guinea pig.

RESULTS. *Experiments on cortical and subcortical localization.* Rhythmic chewing was obtained from points on the anterior and lateral aspects of the cortex in both rabbits and guinea pigs. Bremer's division of the "chewing area" of the rabbit's cortex into three parts giving different types of masticatory response was not confirmed. Opening of the mouth and rhythmic chewing, as described below (p. 170), were the only results obtained from stimulation of the cortex. From animal to animal the points which when stimulated evoked chewing most readily tended to vary in position within a fairly large area, but with stimuli of minimal strength the region was definitely circumscribed. The course of the subcortical path which was found was grossly the same in rabbits and guinea pigs. It coincided with that described for rabbits by Miller (1920) and will therefore not be dealt with here.

The six guinea pigs of the "survival" experiments were kept for the following periods of time, not including the day of operation: 2 animals for six days, 1 for five days, 3 for four days. The animals were killed because of the development of general weakness, due to difficulties in feeding. Stimulation of the brain stem of these six guinea pigs gave various movements of the head, neck, trunk, and limbs, but at no level was any chewing elicited.

Experiments on the mechanism of chewing. A record was taken from an intact, unanesthetized rabbit, eating lettuce. This is shown in figure 1*A*. Chewing is at a rate which remains fairly regular at about 4 per

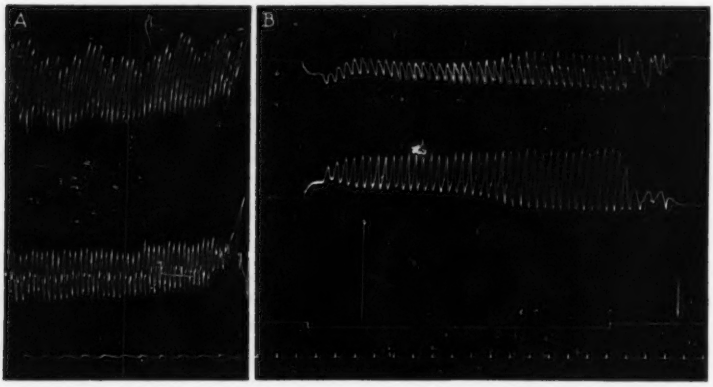

Fig. 1*A*. Record of normal chewing. Unanesthetized rabbit. Lateral jaw movements above, upstroke of lever indicates jaw movement to left. Vertical jaw movements below, upstroke of lever indicates jaw opening. Rate of chewing 4 per second. Simultaneous ordinates at right. Time recorded at one-second intervals.

Fig. 1*B*. Chewing, similarly recorded, elicited by cortical stimulation at rate of 36 per second. Rate of chewing 2.4 per second. Lower chewing record is 0.08 cm. to right of upper. The signal marks the time of stimulation.

second. The initial movement in chewing is opening of the mouth, and the beginning of contraction of the pterygoids coincides with that of the masseters and temporals.

On stimulating the cortex with a low frequency, 1 to 9 stimuli per second, contraction only of the jaw-opening muscles is obtained. The jaw movements obtained at these rates of stimulation can best be described as a "fine" type of activity in which a small single contraction results from each stimulus. These fine movements are to be distinguished from rhythmic chewing. Direct examination of the masseter reveals no contraction at these low frequencies.

Increase in rate of stimulation above a threshold frequency initiates

rhythmic chewing (fig. 2*B* and table 1). The threshold for cortical stimulation varies usually between 9 and 20 stimuli per second, though in one instance it was as low as 4 per second. Chewing from stimulating sub-

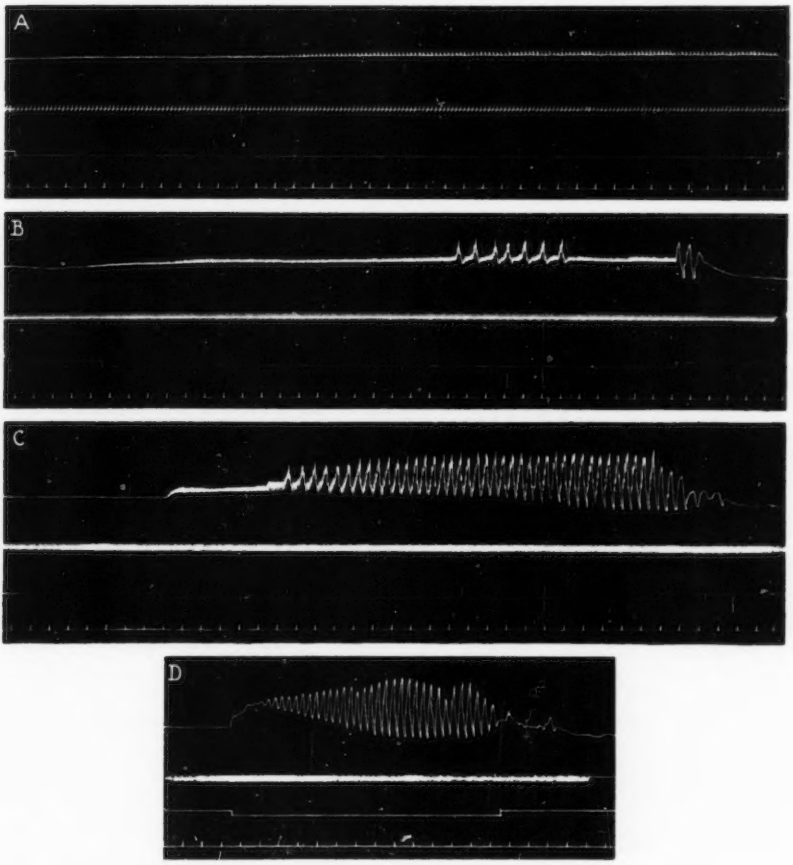

Fig. 2. Vertical jaw movements obtained by cortical stimulation with increasing frequencies. Upstroke of lever indicates jaw opening. Second line is the record of the frequency of stimulation. Signal and time records as in figure 1.

A. Frequency 4.6 per second. Response at same rate.

B. Frequency 14 per second. Fine movements at identical rate merging into rhythmic chewing at rate of 1.1 per second.

C. Frequency 19 per second. Fine movements at 19 per second. Rhythmic chewing 2.1 per second.

D. Frequency 64 per second. Initial prolonged rise of lever (jaw opening). Rate of chewing 2.8 per second.

cortical regions was more readily elicited, frequencies as low as 2 to 3 per second being adequate in one experiment. The threshold remained relatively constant for long periods.

In rhythmic chewing, as in the fine type of jaw movements, the initial response is always jaw opening (upstroke of lever). The fine contractions of the jaw-opening muscles are now superimposed upon the larger excursions. A quick contraction of the jaw-closing muscles (downstroke of lever) follows the opening. Analysis of the records indicates that the rate of this contraction remains constant regardless of the rate of chewing.

TABLE 1

Relationship between rate of stimulation and rate of chewing response per second

RATE OF STIMULATION PER SECOND	DATE OF EXPERIMENT													
	January 13		January 16		February 8	July 20	February 16		March 1		June 13		June 27	
	C	S	C	S	C	S	C	S	C	S	C	S	C	S
0– 5		1.9		1.9		1.8				2.0		1.3		1.7
5– 10			1.0	3.2		2.0		2.3		2.2		1.3	1.8	1.9
10– 20	1.5	2.7	1.4		2.1	2.2	2.1	2.7		2.1	1.3	1.4	1.9	1.9
20– 30	2.9	2.2	2.7				2.3	2.5	1.2	2.5	1.3		2.1	1.7
30– 40	2.3	2.7	2.2		2.3	2.0		2.4	2.2	2.0	1.5	1.0	2.0	1.7
40– 50					1.8	2.4	1.8	2.1	2.0	2.5				
50– 60	2.5				2.4	2.2					2.2		1.9	2.1
60– 70		3.4	2.4			2.5	2.9		2.1	2.6				
70– 80		2.9	2.1		2.6	2.4			2.6	2.7	2.2	1.4		1.7
100–124		2.7	2.8			2.4			2.7					
125–250		2.9	2.3			2.2					1.1		i	2.1
150–200			i								i			
250				3.2		2.0								
401				i										

I = Inhibition.
C = Rate of chewing for cortical stimulation.
S = Rate of chewing for subcortical stimulation.

Examination of the lateral movements of the jaw, as indicated by the upper record in figure 1*B*, shows that the period of action of the pterygoid muscles coincides with the contraction of the jaw closers. During the phase of jaw opening, the pterygoids remain relaxed.

Further increase in frequency augments the rate of chewing. The rate depends upon increase in the speed of the jaw-opening phase (fig. 2*C*). At frequencies above approximately 20 per second the rate of chewing is relatively independent of the rate of stimulation (table 1). At higher frequencies a prolonged phase of jaw opening is present before chewing starts (fig. 2*D*). At rates above 150 per second the movements become irregular

and slow, and are frequently entirely absent. At the lower frequencies of stimulation the small contractions of the jaw openers are present during the contraction phase. At somewhat higher frequencies, they are still present during jaw opening, but in certain cases cannot be seen during the jaw-closing phase.

There is a definite relationship between the latent period of rhythmic chewing and the frequency of stimulation. Figure 3 indicates the typical shape of the curve obtained by plotting the duration of the latent period against the frequency of stimulation. At higher frequencies the period measured from the time of the stimulus to the beginning of the phase of jaw opening was taken as the true latent period.

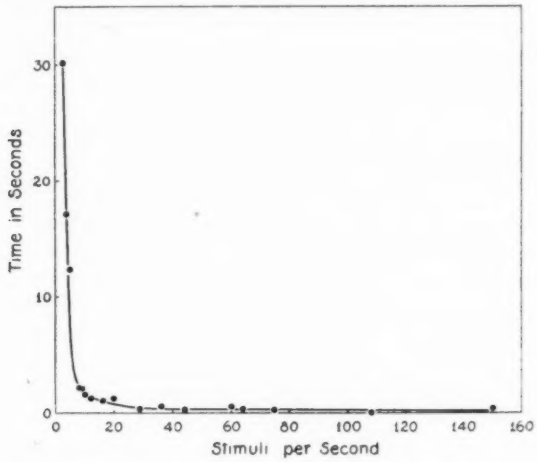

Fig. 3. Abscissae: latent period of rhythmic chewing in seconds. Ordinates: Frequencies (shocks per second) of stimulation of subcortical regions.

A few experiments were performed in which the intensity of the stimulus was varied, but the frequency kept constant. In these experiments little or no change was observed in the rate of chewing, although the intensity was varied as much as 200 per cent.

After-discharge was often present on both cortical and subcortical stimulation. This ranged usually from 0.1 to 3 seconds in duration, and was longer when the cortex was stimulated. It was very variable and bore no relationship to the frequency of stimulation. In one rabbit under light anesthesia, chewing continued for 9 seconds after cortical stimulation had ceased.

In these acute experiments the response on cortical stimulation differs from that on subcortical stimulation as follows: in the former the latent

period of rhythmic chewing is longer, the frequency threshold is higher, chewing is slightly slower, and the after-discharge tends to be longer. Frequently response from subcortical stimulation was obtained when the cortex was entirely inexcitable.

DISCUSSION. Jaw opening, with inhibition of the powerful jaw-closing muscles, as the usual response on cortical stimulation, has been discussed by Sherrington (1906 and 1917). He has found that there is a large representation in the cortex of the function of jaw opening, whereas jaw closing is not regularly obtained. He has been able to show that the principle of reciprocal innervation operates in the case of the jaw-closing and jaw-opening muscles. In view of these facts it is reasonable to suppose that in the present investigation inhibition of the jaw closers always accompanied the first phase of rhythmic chewing, which is opening of the jaws. This would account for the prolonged period of jaw opening which precedes chewing at the higher frequencies of stimulation, when inhibition of the jaw-closing center due to summation of stimuli from the cortex might be greater. The total absence of jaw movements at still higher frequencies does not lend itself to a simple explanation.

The presence of the fine separate contractions of the jaw-opening muscles during the closure of the jaw at the low frequencies of cortical stimulation indicates the absence of reciprocal innervation in such cases. At slightly higher frequencies, still below the tetanus fusion rate of the jaw openers, no signs of activity of these muscles can be seen during the jaw-closing phase in certain cases. This would suggest that for the jaw muscles reciprocal innervation is a function of frequency of stimulation. The method employed here was not sufficiently delicate, however, to decide this point.

As shown above, the rate of jaw opening in rhythmic chewing (p. 172) and its latent period (p. 173) are functions of the frequency of cortical stimulation. This is in keeping with the idea of the building up of a central excitatory state at the jaw-opening center and of a central inhibitory state at the jaw-closing center. From the data presented here no inferences can be drawn as to the nature of the process. The long opening phase and the irregularities in rhythmic chewing seen at the higher frequencies of stimulation suggest relatively increased inhibition of the jaw closers at these frequencies. The difference between cortical and subcortical stimulation suggests that in the former case certain intracortical inhibitory fibers are being stimulated. The cessation of stimulation removes their inhibitory effect and a longer after-discharge is observed.

In attempting to explain the rhythmic character of mastication, one cannot assume the presence of an intermediary center situated in the midbrain. Bremer (1923) believes this supposition to be unnecessary. The results obtained on the chronic experiments reported above support this

view, although it may well be pointed out that the period allowed for degeneration of the cortical paths was too short. In view of the entirely negative results, this point becomes less significant. Magoun, Ranson and Fisher (1933) have recently shown that the substantia nigra can no longer be regarded as an intermediary center in the corticofugal pathway for lapping, in cats. They stimulated the substantia nigra, after unilateral degeneration of this corticofugal path, and found complete absence of these masticatory reactions on the operated side. On the unoperated side, lapping movements were easily elicited.

If no mid-brain "center" exists, one must suppose the cortex to be acting directly upon the motor nuclei of the hind-brain. The cortical influence is evidently inhibitory for the jaw closers. Sherrington has pointed out that this cortical inhibition offsets a tonic state of excitation

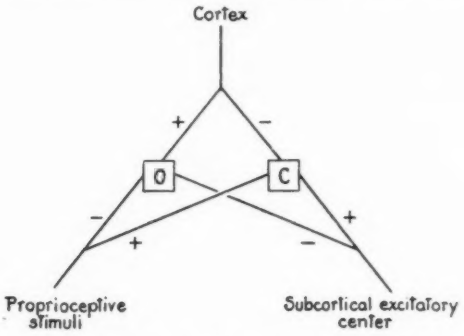

Fig. 4. Schematic representation of the mechanism of mastication. O = jaw-opening center. C = jaw-closing center. Plus and minus signs indicate excitatory and inhibitory effects respectively.

present in the hind-brain center and controlled by "some cranial mechanism seated between cerebrum and bulb." He also suggests that the local proprioceptive stimuli represent another source of excitation.

This tonic excitatory effect is indicated by the constant rate of jaw closing found in the experiments reported. There is definite evidence that proprioceptive stimuli tend to excite jaw closing, for sudden stretching of the jaw-closing muscles initiates a quick contraction (Rioch and Lambert, 1934).

A purely hypothetical and schematic representation of the chewing mechanism is indicated in figure 4. This is presented in the simplest form possible and could be greatly varied. Stimulation of cortical paths relaxes the jaw closers and excites the jaw openers. At low frequencies sufficient excitation of the jaw openers is not present to initiate chewing nor is there sufficient relaxation of the jaw closers. This would account for the

frequency threshold. Increase in frequency initiates jaw opening. This immediately gives rise to proprioceptive stimuli which facilitate the excitatory effect upon the jaw closers of the hypothetical subcortical center, producing sufficient excitatory state to initiate a contraction. As soon as the jaw is raised, the proprioceptive stimuli cease and relaxation of the jaw closers again occurs, together with contraction of the jaw openers from cortical or subcortical excitation.

SUMMARY AND CONCLUSIONS

The localization of central mechanisms for rhythmic chewing is essentially the same in rabbits and guinea pigs. Evidence against a suprabulbar "chewing center" in guinea pigs is presented.

An analysis is given of rhythmic chewing, as obtained by cortical and subcortical stimulation, with stimuli of variable frequency and intensity.

There is a frequency threshold for rhythmic chewing, below which only jaw-opening movements are obtained at rates identical with the rate of stimulation. An upper threshold exists above which inhibition of chewing takes place.

The rate of jaw opening and its latent period are functions of the frequency. The rate of jaw closing is relatively constant.

Evidence is presented to suggest that the reciprocal innervation of the muscles of mastication is also a function of the frequency.

An after-discharge is at times present, longer on cortical than on subcortical stimulation.

Evidence is given for supposing intracortical inhibitory fibers to be present.

A theoretical consideration of the mechanism of mastication is presented.

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ASPHYXIA OF THE FROG'S KIDNEYS

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In studying the effects of lesions of the central nervous system of frogs upon their water balance it was found that any brain injury that interfered with the breathing also stopped the formation of urine. Other injuries to the brain did not reduce the rate of water excretion. That the interruption of urine production was possibly due to lack of oxygen was indicated by the fact that in temperatures low enough to oxygenate the blood through the skin alone, urine continued to be formed in spite of the injuries. This view has now been confirmed by experiments in which frogs were deprived of oxygen.

The mechanism of the anuria caused by lack of oxygen has also been investigated with the surprisingly simple result that all glomerular circulation ceased immediately upon the withdrawal of oxygen, resuming again after its restoration. Analysis of this response gave results that will be described below.

The double nature of the circulation in the amphibian kidney has interested physiologists for many years. Practical use of the arrangement of the blood vessels for experimental purposes was first made by Nussbaum (1878a). Upon ligation of the renal arteries no urine was produced. Bainbridge and Beddard (1906) found that in an atmosphere of oxygen certain pathological changes in the renal tissue that followed this ligation were prevented. Perfusion of the kidney's blood vessels through both the arterial and the renal-portal systems was first carried out by Cullis (1906). Cullis (1908) showed that omission of oxygen from the perfusate slowed the rate of urine formation to one-third of its initial rate. When Bainbridge, Collins and Menzies (1913) perfused both systems with boiled Ringer's solution, urine was still obtained, and this seemingly diverted attention from the obvious necessity of studying the effect of lack of oxygen upon the normal frog's renal circulation.

It was concluded from the experiments of Nussbaum (1878 a, b) and of Bainbridge and Beddard (1906) that urine might form when the renal arteries were ligated if urea solutions were injected. This observation

was possibly explained by the experiments of Tamura (1927b, 1928), Richards and Walker (1927), and others, who found that glomerular capillaries might receive fluid from the renal-portal system or other blood vessels even though the renal arteries were occluded. Obviously the "Nussbaum experiment" is not usually fully accomplished by ligating the

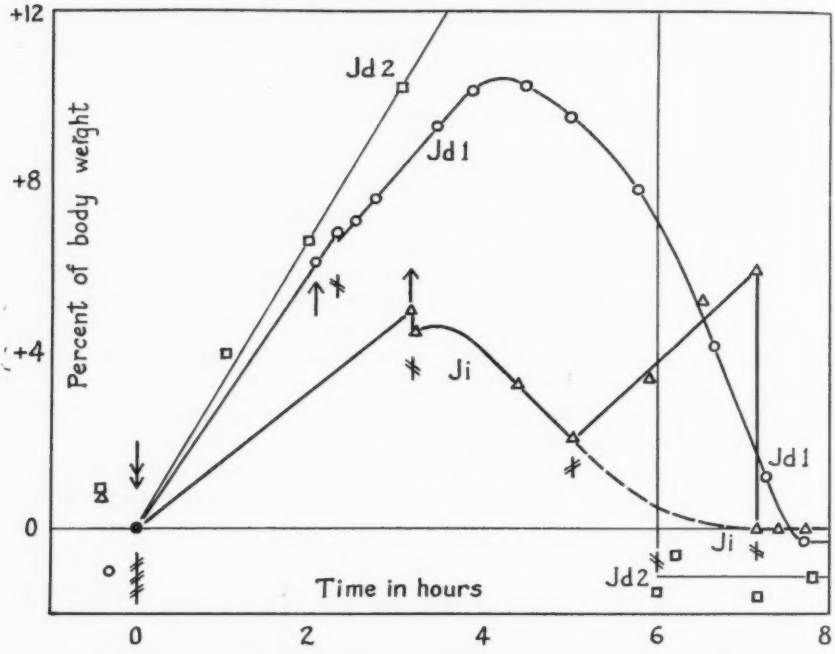

Fig. 1. Changes of body weight and of urine formation in three frogs at 21°C., all of whose cloacas were bound at zero time. *Jd 1*, frog subjected to lack of oxygen for 2.1 hours with cloaca bound for 2.3 hours, evidently no urine formed during this period; in 7.5 hours the original body weight was finally attained. *Jd 2*, the same frog one week later, kept in room air, the cloaca being bound for 6.0 hours, when urine was collected. *Ji*, frog subjected to lack of oxygen for 3.1 hours, after which one drop of urine was collected from the cloaca; after partial recovery the cloaca was again ligated at 5.0 hours, and at 7.1 hours an amount of urine that indicated compensatory diuresis was collected.

renal arteries. Apparently it is accomplished accurately and without surgical intervention, as will be described, by the simple removal of oxygen.

RATES OF URINE FORMATION. All the data in the present paper were obtained upon the species *Rana pipiens*. Rates of urine excretion were measured by ligating the cloacas of frogs so that urine when formed had to remain in the bladder. The frogs were kept at 20° to 24° C. and were

weighed at intervals, the gain in weight being proportional to time, as is shown in figure 1. When put into nitrogen, frogs gained at similar rates, but upon removing the cloacal ligature no urine issued (28 frogs) and the body weight continued to increase for some time after air was again furnished (fig. 1). Urine was always expelled from the bladder quantitatively, even by limp anesthetized frogs, when the body was appropriately squeezed, so that the result was not due to any failure to expel the urine. In addition a number of frogs were killed at the end of asphyxia and their bladders were ascertained by dissection to be empty. In an atmosphere composed of 30 per cent carbon dioxide and 70 per cent nitrogen the result was the same (6 frogs).

When 4 per cent oxygen was present the frogs (at 20° C.) formed urine at the usual rates (3 frogs). Even in 3 per cent oxygen some urine was formed (2 frogs), and it was apparent that very little oxygen was required to maintain normal function of the kidneys. At 5° C. the nitrogen obtained from ordinary supply cylinders contained enough oxygen to maintain the normal rates of urine formation (4 frogs), which at that temperature were slow, however.

If room air was again supplied to the frogs after treatment with nitrogen for 2 to 3 hours at 20° C., the increase of body weight was soon checked. In a few instances this happened within one hour, but usually 1 to 4 hours were required. Then the body weight rapidly diminished until the original level was reached (fig. 1). If, however, the cloaca were tied so that urine was retained in the bladder, the body weight increased at its original rate, and upon removing the ligature from the cloaca an amount of urine escaped that was larger than the increase of body weight during the period of ligation (fig. 1). This was observed in 11 frogs, most of them having their cloacas ligated twice, once during the asphyxia and once during the recovery period. It was obvious that the lack of oxygen did not modify the rate of influx of water through the skin; it did completely stop the output of water through the kidneys.

Attempts were made to overcome the anuria by inducing diuresis. For this purpose either water (3 frogs) or 1 per cent urea solution (6 frogs) or 10 per cent urea solution (2 frogs) was injected into the lymph sacs in large amounts just before the oxygen deprivation. In no case was urine formed as long as oxygen was denied the frogs. In control frogs similarly injected that remained in air considerable diuresis developed, as was previously shown (Adolph, 1927), and in the experimental frogs the rates of urine formation rose considerably above the normal as soon as recovery from the lack of oxygen supervened. Evidently the kidneys were not permanently damaged, yet during asphyxia no diuretic material would induce the kidneys to form urine.

Other modes of inducing asphyxia were tested. As already mentioned,

the cessation of breathing induced anuria. This was true only at temperatures of 20° to 27° C.; at 5° C. the cessation of breathing did not prevent urine formation at the usual rate, for as is well known a frog at such temperatures receives all the oxygen that it requires through the skin. No one of a variety of surgical operations upon the central nervous system influenced the kidneys directly, and even those operations that stopped the breathing had no effect upon water excretion at 5° C. (Adolph, 1933).

Mixtures of carbon monoxide and air induced partial asphyxia, so far as could be judged from the rates of urine formation. Five frogs were subjected for two hours to atmospheres containing about 5 per cent of carbon monoxide and 95 per cent of air. In two of them no urine was formed, while in three of them about half of the gain in gross body weight was accounted for as urine. These frogs did not become prostrated, and recovered much more rapidly than those in nitrogen. In fact it was difficult to distinguish the frogs subjected to carbon monoxide from those kept in air except by the partial suppression of urine formation.

Under some circumstances frogs were prostrated by subjection to lack of oxygen. Even when all reflexes had disappeared subsequent recovery frequently occurred. At higher temperatures breathing usually ceased, with or without prostration. But in winter frogs at 20° C. breathing persisted and many reflexes remained; yet these frogs too exhibited complete anuria.

Renal blood flow. To find the cause of the anuria produced by asphyxia it was natural to examine the blood flow in the kidneys. Each frog for observation was pithed bloodlessly by clamping the brain in a hemostat. The frog was put in a gas-tight chamber through which a rapid flow of room air was maintained; one kidney was spread out by grasping with a clamp or hook the mesentery attached to it, and the flow in its blood vessels was observed by transmitted light.

As soon as air was driven from the chamber by nitrogen, the blood ceased to flow in all the glomeruli that could be seen. The vessels of the renal-portal system continued to conduct blood at the usual rates, but flow in the entire renal arterial system promptly stopped. The first glomeruli usually became quiescent in less than one minute; the last glomeruli stopped in two to four minutes, depending upon the rate of gas flow and the size of the chamber used. Of 49 observations made, some glomeruli continued to conduct blood in only one instance, this one being the first of four administrations of nitrogen to the same frog. Stoppage was observed in 5 female as well as in 21 male frogs.

When hydrogen was substituted for nitrogen the flow of blood in the glomeruli likewise ceased (3 times in one frog). In a mixture of 30 per cent carbon dioxide and 70 per cent nitrogen the result was the same (2 frogs).

When air displaced the nitrogen from the chamber the return of activity in the glomeruli was equally prompt. The first glomeruli showed blood flow within one minute, the last that recovered at all did so within three minutes. The flow in any given glomerulus was often slow for about one minute, but very promptly attained its full velocity. Commonly the last glomeruli to stop were the first glomeruli to resume their flows.

Mixtures of air and nitrogen were tested to see what was the least tension of oxygen required to prevent asphyxia of the kidneys. In 4 per cent of an atmosphere of oxygen the glomerular circulation always remained normal (4 frogs). In 3 per cent of oxygen the circulation was unchanged except that in one frog one group of glomeruli became inactive (3 frogs). In 1.5 per cent of oxygen at least half of the glomeruli became inactive (2 frogs), and hence it was evident that this was approximately the critical tension for frogs in good circulatory condition with viscera exposed. The intact frogs had shown partial urine formation in 3 per cent oxygen, but in these animals the viscera were not exposed. It was interesting to find in frogs that had already been subjected to nitrogen that the renal arterial and glomerular circulation was fully restored when this gas was displaced by a mixture containing only 3 per cent of oxygen.

The effects of 1 per cent carbon monoxide (2 frogs) and of copious intravenous doses of cyanide (3 frogs) were tested in relation to the renal blood flow. Neither had any significant effect in reducing the blood flow in the presence of air. This result indicates that sufficient oxygen to keep the renal glomeruli open was furnished to the kidney when either the supply or the utilization of oxygen was markedly diminished. Probably neither of these influences produced any greater degree of asphyxia than the subjection to 4 per cent oxygen. The pithed frogs in all experiments were obtaining their oxygen supply to the blood stream through the surfaces of skin and exposed visceral organs, since no breathing occurred in any of them. It is well known that this supply is almost sufficient for all purposes in resting frogs, especially when kept at moderately low temperatures; in addition the operated frogs had more surface exposed to the entrance of oxygen.

The general circulation. To find why the blood flow stopped in the glomeruli, it was first necessary to study the changes in general systemic circulation. For this purpose one aorta was cannulated in each of four frogs, the cannula being connected to a small mercury manometer. The whole was then inclosed in a gas-tight chamber through which either room air or nitrogen was passed. The changes of mean arterial pressure and of pulse rate are shown for one frog in figure 2. Every time oxygen was removed the pressure fell by about 40 per cent of its initial value. The recovery in each instance was smaller than the fall. The heart rate fell in parallel with the arterial pressure, indicating that the heart itself

was responsible for most of the change of pressure. If the response to asphyxia had been primarily in the peripheral blood vessels, the heart might have been expected to compensate, at least temporarily, by increasing its rate.

A reason for not crediting the glomerular failure to the central change is that the arterial pressure under nitrogen was sometimes higher than it subsequently was with oxygen present. Moreover, the glomerular circulation ceased before the arterial pressure had fallen very much. But it might conceivably be that the renal vessels responded to the rate of fall of arterial pressure and not to its absolute value. This point was checked by producing fall of blood pressure in other ways. Administration

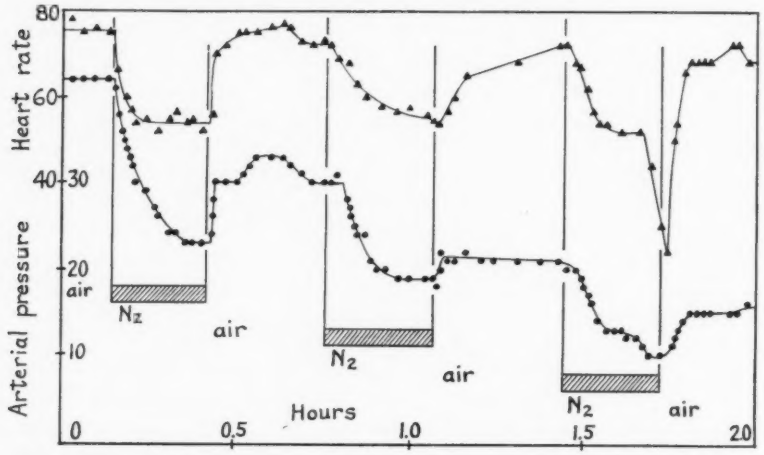

Fig. 2. Changes of mean arterial pressure, in millimeters of mercury, and of heart rate, in beats per minute, in frog *Ec* at 26°C. In alternate periods room air and nitrogen were administered.

of amyl nitrite (2 frogs) to the frogs while their kidneys were under observation slowed the entire blood flow greatly, but did not stop the circulation in most of the active glomeruli. Stimulation of the vagi by minimal tetani (2 frogs), so that the arterial pressure and the heart rate were diminished by amounts at least comparable to those produced by lack of oxygen, failed likewise to embarrass seriously or persistently the blood flow through the glomeruli.

If the stoppage of blood flow in the renal artery were due to insufficient blood volume, it might be expected that injection of fluids into the blood stream would prevent the reaction to asphyxia. That this was not the case was indicated in the experiments (mentioned before) where 10 per cent urea solution was administered intravenously. The renal arteries

stopped just as readily as without it. Other experiments in which 0.6 per cent sodium chloride solution was infused just before the administration of nitrogen gave the same result. This evidence is not particularly significant, since a failing heart might easily render an increase of circulating volume of no avail; but if the outcome had been contrary the interpretation would be quite different.

The renal arterioles. Study of the renal arteries and their branches gave evidence of the exact location of the block to glomerular blood flow. Upon administering nitrogen the flow usually stopped in the main renal arteries. When this occurred it indicated of course that the flow ceased in *all* the glomeruli. But these arteries remained open and the blood within them moved backward and forward with each pulsation of the heart. Hence there was no occlusion of arterial vessels between them and the heart, a fact that was also ascertained by observing the vigorous blood flow to all the neighboring organs. When the small arterioles on the ventral surface of the kidneys were observed, however, the picture was in strong contrast. As asphyxia developed their lumina became narrower and narrower, until the flow of blood ceased and no blood was contained in the vessels, which now stood out as ghost-like ribbons over the red tissues of the kidney. It seemed apparent that these renal arterioles actively constricted and thus shut off the flow of blood to the glomeruli. The afferent vessels leading to individual glomeruli also emptied of blood corpuscles; hence it appears that they too responded to lack of oxygen by active constriction. That the afferent vessels are capable of independent constriction in response to local mechanical pressure was found by Okkels (1929), and in response to local applications of concentrated salt solutions was observed by Ebbecke and Jäger (1933).

The branches of the renal arterioles that supply blood to the mesentery lateral to the kidneys and to the Müllerian ducts were also observed. These arterioles and the larger branches of the renal arteries leading to them could be seen in great detail, since no tissues that were not wholly transparent lay above or below them. In them the flow of blood diminished slightly when asphyxia developed. Their lumina always remained patent, and the blood usually persisted in flowing through them. Whether it did or not depended upon whether they branched from the renal arterioles centrally or peripherally to the points of complete constriction. Hence it is to be concluded that the inhibition of blood flow was not due to the glomeruli themselves but to the renal arterioles. The response was often limited to the truly renal arterioles, thus not including the separate arterioles that led to the oviduct. That venous stasis was in no way concerned was indicated by the perfect continuance of blood flow in the renal-portal vessels, and by the fact that the glomeruli usually emptied completely of corpuscles.

There was no evidence that the blood flow ceased anywhere else in the body than in the renal arterial system. The surfaces of other tissues that were visible showed blood flowing, and the renal-portal circulation was in no way interfered with. This might suggest that the cessation of flow in the kidney's arteries was in some way connected with the fact that the kidney has a double blood supply. One other organ in the frog has a double blood supply, the liver; no observations were made, however, upon the flow of blood in the hepatic artery during asphyxia.

Nervous control. The next step was to find whether nervous reflexes were involved in the constriction of renal arterioles. This was done by destroying the fine nerve branches that go to the kidneys. These branches are often referred to as the splanchnic nerves (Bieter, 1930), but it was not considered satisfactory to cut merely the large trunk that is termed the splanchnic nerve by Gaupp (1899), since this does not supply the kidney as regularly or extensively as the small branches located posteriorly. Therefore the sympathetic ganglia of one side, clinging to the walls of the dorsal aorta, were removed. In spite of this denervation (3 frogs) the circulation in the glomeruli stopped in response to lack of oxygen. Hence the response was of local origin in the kidneys, either due to the sudden fall of blood pressure, which was shown before to be unlikely, or to the asphyxia of tissues, perhaps of the arterioles themselves.

The denervation was in each frog fully checked at autopsy, and it was ascertained that every sympathetic ganglion had been removed from the kidney region of the side studied. It would be possible to suppose that other nerves go to the kidneys, perhaps perivascular ones. Concerning forms of innervation other than the sympathetic trunks to the frog's kidney there appears to be no information (Hirt, 1930). The possibility is not ruled out, of course, that the denervated kidney still employs the intrinsic nerve fibers in the asphyxial response, possibly in axone reflexes.

There was in one frog out of three some suggestion that after denervation the response was not so complete as usual. Three glomeruli and their arterioles out of about 20 glomeruli that were being watched maintained a very slow circulation during lack of oxygen. It is probable that this instance was not significant, since glomerular activity had been also found in the kidney of one frog where no denervation was performed. In this experiment the mechanism of resumption of blood flow was elucidated; for those glomeruli that had a slow circulation regained their original circulation immediately (within 20 seconds) after oxygen was administered, while those glomeruli that were stopped altogether required 1 to 3 minutes for the return of circulation to them. Evidently the return of blood flow was at least not characteristic of a generalized nervous regulation.

Local nature of the response. The possibility was now raised that loci

within the kidneys, perhaps each arteriole itself, had to receive oxygen before it reopened. The oxygen might conceivably be brought either by diffusion from the kidney surface or by the renal-portal blood flow, or by both. Otherwise a distinct difficulty would exist as to how an asphyxiated arteriole would ever again recover. Further evidence as to whether or not each arteriole or each afferent vessel responded independently to the lack of oxygen hence had to be obtained. From one point of view evidence of independence might have appeared not to exist, since it was a general experience that kidneys subjected to tensions of oxygen between 1.5 and 4 per cent of an atmosphere either responded in nearly all their glomeruli or did not respond in any glomeruli to the asphyxia. Possibly this could be indicative of a nervous control superimposed upon or reinforcing the local sensitivity of the arteriolar walls to lack of oxygen, but this is believed not to be the case.

The local nature of the response became evident when a study of the recovery from asphyxia was made. By what route did oxygen reach the asphyxiated arterioles so that they could again open? Two frogs, one with denervated kidney and one not, were subjected to three successive periods of asphyxia each of 6 minutes' duration, separated by prolonged exposures to room air; just before the middle period of asphyxia the renal-portal vein was occluded by a loose ligature. Hence during this one exposure and recovery no blood was supplied to the kidney, since the dorsolumbar vein of the kidney had been in each case previously tied off. The same length of time was required for the glomerular and arteriolar blood flows to cease as in the other asphyxial periods; in this one exposure the entire venous flow of the kidney also ceased, though pulsations continued in the venous blood. But the recovery from asphyxia required at least 5 times as long when the renal-portal flow was stopped. Instead of the 2 minutes that were normally required for a majority of the glomeruli to regain blood flow, 10 to 14 minutes were required, in the denervated kidney as well as in the normal one.

The whole recovery picture in the absence of renal-portal blood flow was in fact a slow motion of the usual recovery. It was very obvious in these instances that within a group of glomeruli supplied by a common arteriole all went into activity at about one time, while distant glomeruli might require twice as much time before activity appeared.

An attempt was made to see whether the asphyxial reaction of the renal vessels could be obtained by removing air from the surface of the exposed kidney. A capsule was lowered over the kidney, a rubber membrane hanging as a curtain down to the tissues. A rapid stream of nitrogen led through the capsule failed to produce any effect (2 frogs). No great significance can be attached to this result, since arterioles are expected to respond to the oxygen tension of the blood bathing them more readily

than to that outside the organ; but a different outcome would have been decisive proof that the contrary was the case.

Another experiment consisted in keeping the kidney and surrounding tissues under the capsule flooded with nitrogen while room air was readministered to each of two asphyxiated frogs enclosed in the gas chamber. Whereas recovery of blood flow in the glomeruli then required about twice the time in which to become nearly completed, yet all the arterial blood flow was restored in spite of the local lack of oxygen. The slower recovery was of course due to the fact that the room air supplied to the frog was diluted by the nitrogen within the chamber and that fewer of the animal's surfaces could aid in oxygen uptake.

Obviously the site of the asphyxia was not superficial but was deep in the kidney. It was located where the renal-portal blood could supply it with oxygen by diffusion, but where diffusion of oxygen either from other blood vessels of the body or from the exposed surface was barely able to suppiy it after a long period of recovery time. If the renal-portal blood flow were to be occluded at the same time that oxygen was denied access from the surface of the kidney it could be expected that no recovery from the asphyxial response would occur.

INTERMITTENCY. Observers of the circulation in the frog kidney have noted that the flow of blood within one glomerulus and within one glomerular capillary may cease and resume over rather short periods of time. The theory was put forward by Richards and Schmidt (1924) that the rate of flow through the glomerulus was a function of the "call for oxygen." Incipient lack of oxygen in a glomerulus or its afferent vessel was suggested as being the stimulus to resumption of flow through the glomerulus. A consequence of this hypothesis would be that lower oxygen tensions should reduce the length and number of periods in which the blood flow ceased.

Observations were therefore made before, during and after subjection to 3 and 4 per cent oxygen. It was disappointing to find no difference in the blood flow through a given glomerulus, even when the flow was highly intermittent and scanty. Other observations were made to compare the flow immediately after complete asphyxia with the later flow when the supposed "oxygen debt" might have been paid off. Again no difference in the direction anticipated was found. No truly quantitative measurements were made of intervals and rates of blood flow, but it seems certain that the differences that could be measured would be insignificantly small.

The fact that intermittency in flow through glomerular capillaries sometimes occurs is undisputed. The present work has given the impression that intermittency is not of common occurrence, and it is best seen in animals whose circulations have been somewhat interfered with. This view was previously arrived at by Tamura (1927a), Brühl (1928), and

others. Intermittency is said to be abolished by section of the splanchnic nerves (Bieter, 1930).

It might be supposed that the spontaneous stoppage of flow in a glomerulus over a longer period, of minutes or hours, would be influenced by lack of oxygen. The observations show, however, that in 4 per cent oxygen the same glomeruli remained open or closed respectively both before, during and after the treatment. None opened up, none permanently closed down, except very occasionally. In absence of oxygen glomeruli that were closed before the asphyxia did not open up after the treatment. In general, efforts to link asphyxia with local blood-flow were unsuccessful; only in the kidney's arterial system as a whole have influences of asphyxia been found.

If intermittency or patency of glomerular blood vessels were related to oxygen shortage, then it might be that polyuria would result from partial lack of oxygen. In other words, if as has been suggested by some investigators diuresis results from increased glomerular blood flow, and increased glomerular blood flow results from local shortage of oxygen, more water might be excreted. Measurements of urine formation by normal frogs kept in 4 per cent oxygen (4 frogs) showed no differences from that by frogs kept in air or in pure oxygen.

Undoubtedly the asphyxial hypothesis of glomerular intermittency could be modified to fit the facts here presented. But the hypothesis would at present be wholly gratuitous, since there is no positive evidence in favor of it.

ALBUMINURIA. Urine collected from frogs that have been subjected to lack of oxygen contained no protein. This urine was sometimes found in very small amounts, and presumably represented urine formed just before the asphyxia took effect; but it is possible that occasionally a few glomeruli maintained spasmodic blood flow sufficient to form some urine.

Urine collected during the two or three hours of recovery likewise contained no protein. Such periods of collection usually began as soon as the frogs were resupplied with room air. This was contrary to what previous descriptions of renal physiology of the frog were supposed to indicate.

In five frogs tests for albumin (by nitric acid and by sulfosalicylic acid) were made upon two successive urine samples collected one during and one after the asphyxia, likewise with negative results. Injections of water and of urea solutions under the skin failed to promote albuminuria.

Urine formed by frogs that were subjected to 4 per cent oxygen (3 frogs) contained no protein. That produced by frogs while in 5 per cent carbon monoxide and air (3 frogs) was likewise free of protein.

It might be supposed that the maintenance of the renal-portal circulation prevented the onset of albuminuria. But the portal circulation did not

prevent complete asphyxia, which has been generally believed to be the decisive factor. Chloride continued to appear in the urine in the usual small concentrations after asphyxia, showing that the urine which formed approached in no sense to the composition of a filtrate of plasma. Hence there is no apparent relation between these experiments and Starling and Verney's (1925) experiments with cyanide in dogs.

Evidence for the production of albuminuria by asphyxia has been indirectly adduced from observations on frogs (*e.g.*, Ekehorn, 1931). It is not difficult to show, however, that none of this evidence is of a decisive nature. Now that the experiment of subjecting frogs both to complete lack of oxygen and to low tensions of oxygen has been carried out, this evidence becomes of no account. It is not true that "asphyxia is a frequent cause of glomerular albuminuria in frogs" (Ekehorn). Hence generalizations based upon asphyxia in frogs fail to support the views expressed for other animals. There appears at present to be no clear evidence for the occurrence of protein in the urine purely as a result of lack of oxygen in the kidneys.

It could be suggested that the closure of the renal arterioles in asphyxia is a response that prevents a loss of protein from the plasma that would otherwise occur through damaged capsules. But there seems to be no way of proving that the capsular membranes are damaged since there is no detectable after-effect of the asphyxia. However attractive, such a hypothesis is without foundation.

It was reported by Nussbaum (1878b), and more recently by Tamura (1927c) and Hayashi (1927) that albuminuria resulted when a foreign protein was injected into the lymph sac of a frog; but when the renal arteries were tied off no protein was found in the urine, either foreign protein or plasma protein. From this it appeared, as has been generally believed, that albumin was excreted through the glomeruli if at all. There is, however, a violent contradiction in these experiments. If all the flow of blood through the glomeruli had been stopped, then no urine would have formed and hence there would have been no fluid to test for the presence of albumin. In other words, the Nussbaum experiment offers no apparent way in which the possible rôle of the tubules in elimination of protein can be adequately studied. Evidently circulatory conditions were not adequately controlled in the investigations cited.

A few experiments were performed in which egg white was injected subcutaneously just before the frogs were subjected to lack of oxygen. It was found that whenever urine formed there was much protein in it, whether during partial asphyxia or in the period of recovery from asphyxia.

COMMENT. The separation of blood flow through the glomeruli from that through the tubules has been regarded as a tool useful in determining the rôles of these two portions of the renal structure. It is obvious that

the Nussbaum experiment can now be performed without surgical intervention of any sort. The glomerular blood flow alone is interrupted by subjecting a frog to lack of oxygen. It must be borne in mind, however, that in contrast to the ligation procedure the carriage of oxygen by the renal-portal blood is here stopped, even though no effects of this lack of oxygen in the renal-portal blood have thus far come to light.

However, the Nussbaum experiment has answered practically none of the questions that it was once expected to answer. The only conclusion that can be drawn from it is the non-the-less important one that no urine forms when no blood flows through glomeruli. This point has been, naturally, thoroughly confirmed by the present experiments. The reason why urine forms only in the capsules may easily be the simple one that only in glomerular capillaries is the pressure high enough to squeeze fluid from the blood plasma. In any case there appears to be no secretion of water into the tubules of the frog's kidney. On the contrary it seems to have been demonstrated that larger amounts of water are excreted by the frog when the renal-portal blood flow stops (Woodland, 1923; Miyamura, 1927). In apportioning elimination and absorption of water between capsules and tubules probably the most important factor is that the pressure of the blood in the glomerular capillaries is five or six times that in the capillaries supplied by the renal-portal system (Hayman, 1927, 1928). Care was taken to find whether extreme conditions that tended to promote diuresis would allow some urine to be formed during asphyxia. It was originally supposed by Nussbaum (1878a, b) that solutions such as 10 per cent urea allow of urine formation when the renal arteries are tied off, but this formation of urine has been tentatively explained (Tamura, 1927b) as due either to incomplete ligation or to back-flow of blood into glomeruli from renal-portal vessels. Evidently no practical means of furnishing fluid and solute that call for excretion can overcome the anuria and ischemia of asphyxia.

The locus of action of lack of oxygen was found in the renal arterioles and afferent vessels. It is worthy of note that direct responses to other stimuli have been found in these same vessels by Okkels (1929) and Ebbecke (1933). That blood vessels susceptible to one sort of direct stimulus should be found susceptible to other sorts seems natural. The intimate conditions that make the renal arterioles more irritable than other arterioles are however still unknown.

Perfusion experiments with the frog's kidney are evidently affected by the asphyxial response. It was noted by Cullis (1908) that the Ringer's solution that was perfused through the arteries required oxygenation to promote rapid urine formation. This fact was interpreted to mean that some secretory activity requiring oxygen was going on; the present experiment furnishes a much simpler explanation. Whether the rate of per-

fusion was also influenced by the oxygenation was not stated by Cullis. Oxygenation and deoxygenation of the perfusing fluid was studied by Bainbridge, Collins and Menzies (1913). They obtained urine even when oxygen-free fluid passed through the arterial glomerular vessels, but evidently this may have been due to failure to preserve the normal pressure relations in the capillary anastomosis. They reported modifications of concentration in the urine due to the factor of oxygen tension; those observations have no counterpart in the present study. The method of Cullis was used by Tamura (1928) who found that renal arterial vasoconstruction occurred when the perfusing Ringer's solution was not fully oxygenated. By comparison with the method of Cullis, his own method of perfusing only the arteries, leaving the renal-portal system supplied with blood appeared to be superior. His investigation comes nearer than any other to anticipating the present knowledge of the asphyxial response in the kidneys. Double perfusion with Ringer's solution containing ox corpuscles was carried out by Brühl (1928). He found that the rate of urine formation was greatly decreased when the solution contained M/1500 KCN, yet the rate of perfusion was not diminished, indicating that no arteriolar constriction occurred, the head of pressure remaining constant. Doubling of the pressure increased the rate of flow of the fluid but did not change the number of active glomeruli, and often did not increase the rate of urine formation.

That the rate of flow of perfusate through the renal arterial system does control the rate of urine formation was demonstrated most recently by Oliver and Shevky (1931). This merely indicates the quantitative rôle of blood flow and pressure within the glomeruli in relation to urine formation of the frog.

In mammalian kidneys the tubules are supplied with blood only from the renal arteries; there is no renal-portal system. Hence if a similar response to asphyxia occurred in the renal arterioles, the tubular blood flow and the glomerular blood flow would diminish or cease in proportion to one another. Obviously no separation of the two portions of the excretory structure could be obtained by this means. There is full evidence, however, in experiments to be reported later, that extreme anoxemia almost stops urine formation in the dog kidney.

SUMMARY

1. In the absence of oxygen urine formation in the frog ceased. At 22°C. only 4 per cent of an atmosphere of oxygen was required to prevent this reaction. The anuria could not be overcome by conditions that ordinarily induce diuresis.

2. Upon examining the blood flow through the kidneys it was found that lack of oxygen stopped the flow in all glomeruli. This was not due

to any property of the general circulation but depended upon constriction of renal arterioles.

3. The response of the renal arterioles was not modified by denervation of the kidney. Subsequent recovery depended chiefly upon the bringing in of oxygen by the blood in the renal-portal system.

4. Intermittent and permanent states of blood flow in the glomeruli were not influenced by oxygen tensions that failed to constrict the renal arterioles.

5. Albuminuria did not result from asphyxia or from the ischemia that was produced by it.

6. The selective effect of asphyxia upon the frog's kidneys reproduced precisely the well-known effects of ligating the renal arteries.

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URETHRAL CLONUS

A Contribution to the Physiology of Ejaculation

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During the course of some experimental work on the physiology of the genito-urinary tract of male dogs a striking rhythmical contraction of the urethra was observed. To detect the possibility of injury to the urethra with resultant extravasation of urine as a consequence to partial extirpation of the prostate, the urethra was distended with urine by manual compression of the bladder and simultaneous occlusion of the anterior urethra. Under these conditions the distended portion of the urethra was seen to display a series of regular rhythmic contractions which persisted as long as the distention was maintained. The present report represents a further study of this phenomenon and its possible relation to the mechanism for the ejaculation of seminal fluid.

Innervation of the urethra. As is well known, three sets of nerves supply the muscular wall of the urethra, namely, the hypogastric division of the lumbar sympathetics, the pelvic visceral branch of the sacral autonomic, and the medullated internal pudic nerve. It is not so generally recognized that the membranous portion of the male urethra (that portion extending from the apex of the prostate gland to the pelvic diaphragm, in length about 5 cm. in an adult dog of 20 kgm.) is surrounded by voluntary striated muscle in the peripheral portion of its wall. In the female dog, the comparable portion of the urethra is surrounded by smooth muscle alone; voluntary muscle in the female urethra is concentrated in a narrow band around the external meatus. This anatomical difference is of importance in explaining a functional difference between the sexes in the dog.

v. Zeissl (1) made the observation which Fagge (2), Elliott (3), and Barrington (4) confirmed, that stimulation of the peripheral cut end of the hypogastric nerve in the dog causes contraction of the urethra. Elliott found that an identical contraction is obtained by injection (sic) of 0.1 mgm. adrenalin. Fagge found that stimulation of the pelvic visceral nerves or of the sacral roots had in most cases no effect on the urethra; in a few cases, strong stimulation however produced a slight contraction of the prostatic portion, and a still smaller contraction of the membranous urethra. v. Zeissl and Barrington, on the contrary, found that pelvic

nerve stimulation produced marked dilatation of the urethra. Electrical stimulation of the peripheral ends of the divided pudic nerves caused a contraction of the voluntary striped muscle surrounding the urethra. This contraction became rapidly fatigued, and unlike the contraction produced by stimulation of the pelvic or hypogastric nerves was abolished by curare (Barrington).

The clonus elicited through distention, which we shall describe has not been clearly recorded although Langley and Anderson (5) probably observed the phenomenon; they state: "The lumbar nerves or the hypogastrics though unable normally to cause micturition, often force a little urine into the upper part of the urethra. In such case, the urethra may contract reflexly. . . . . It is possible that similar contraction should be set up when the seminal vesicles force their contents into the urethra." (loc. cit., p. 103.) "In the rabbit where there is much resistance to the

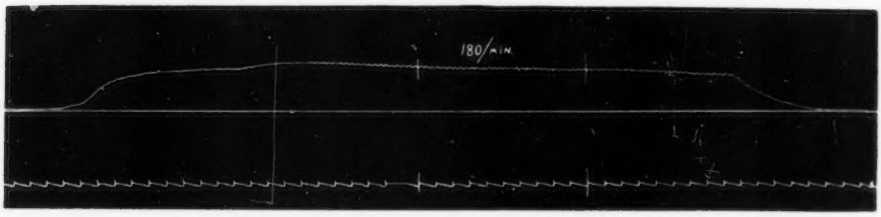

Fig. 1. A catheter was inserted in the anterior urethra of a male dog, so that the eye lay in the membranous urethra and the latter was distended with urine by manual compression of the bladder. The pressure tracing above shows the rhythmical contractions obtained: the contraction rate was 180 per minute and the maximum pressure reached was 46 mm. Hg.

outflow of urine, strong contraction of the bladder drives urine into the seminal vesicles (uterus masculinus) or into the upper muscular portion of the vagina. This usually sets up rhythmic contractions of these organs. The seminal vesicles may drive the urine back again though usually incompletely. Sometimes raising the lower part of the body by the tail compresses the urethra sufficiently to produce this result" (idem., p. 79). It is likely that the reflex mentioned is the same as we present here, but it was not further investigated by these authors and it must be borne in mind that hypogastric stimulation produces contraction of the smooth muscle of the urethra.

EXPERIMENTAL. All experiments were made on dogs under ether anesthesia. The clonic contraction occurred in the membranous urethra, was clearly seen with the unaided eye and easily recorded by motion picture photography. The anterior "lobes" of the prostate were slightly

approximated during the urethral clonus, due to the origin of the urethral striated muscle fibres in this gland, but no other visible or recordable contraction of the gland occurred. It was possible to make the following observations.

a. The clonus was present in the dog anesthetized lightly with ether, and was abolished by moderate or deep ether anesthesia. It was usually elicited when such reflexes as the knee jerk and eyelid contracture resulting from corneal touch were present. It also occurred in the micturating unanesthetized male dog when the anterior urethra was occluded by the fingers and was seen as a *transmitted* clonus in the anterior urethra.

b. The clonus occurred when the urethra was distended with fluid at a pressure of 15 mm of mercury or more. This distention was produced

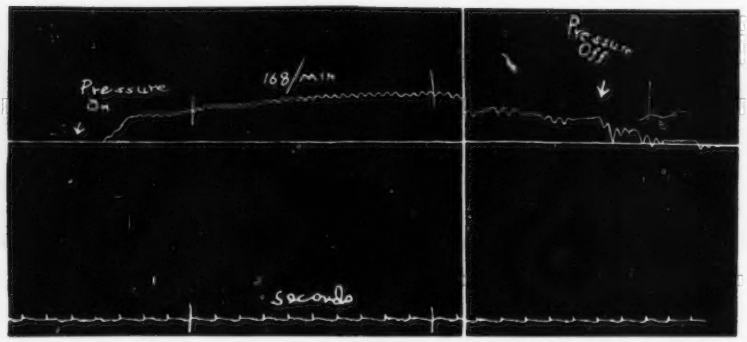

Fig. 2. Same as figure 1, but the clonus was elicited by a much lower pressure, 15 mm. Hg. The clonus persisted more than 2 minutes and a section of the curve has been omitted to show the beginning and end of the tracing. The rate is 168 per minute. Note the "after-discharge" following the release of pressure.

in two ways. In the first method urine in the bladder was forced into the urethra by manual compression, while the urethra was temporarily occluded by the fingers or a ligature. In the second method a large soft rubber catheter (no. 12 Charrière) was inserted in the urethra by making a small incision in the urethra just proximal to the penis bone, a second incision was made in the bladder muscle, large enough to insert a finger to occlude the internal vesical orifice, and the urethra was distended by injecting fluid through the catheter by syringe. The same visible clonus occurred in both conditions. The second method ruled out the possibility that manual compression of the bladder muscle stimulated the hypogastric nerves, since the bladder was left in situ and undisturbed except for the small incision.

c. The contractile waves of the clonus occurred at a fairly uniform rate, about 3 per 1 second. In six dogs the contraction occurred at a rate of 180, 180, 176, 172, 168, 166 per 1 minute respectively.

d. As Barrington and others have found, stimulation with a mild faradic current of one pudic nerve or of the wall of the membranous urethra itself produced constriction of the urethra. This contraction was steady and occurred in the same location as the clonus. The clonus was abolished by section of *both* medullated nerves emerging from the 1st and 2nd sacral foramina running along the superior portion of the pelvic diaphragm and ascending the urethra as far as the prostate gland, that is, the n. pudendus internus. Section of one nerve did not abolish the clonus.

e. The reaction to distention does not occur in female dogs. This is not due to a sex linked reflex but depends on the anatomical difference (see above) between the muscular equipment of the male and female urethra. This is further evidence that the reflex is not a function of smooth muscle, that it occurs in the striated muscle of the male. Further evidence that this is not a smooth muscle response may be gathered from the work of Fagge who presents pressure tracings obtained from the membranous urethra by stimulating the hypogastric nerves. Such tracings showed a steady prolonged smooth elevation of pressure.

f. There is no associated effect on the systemic arterial blood pressure.

g. The clonus is of long duration, and a marked clonus under favorable anesthetic conditions was sustained for a 5 minute period of distention and even then was undiminished.

h. There was found an "after-discharge" of clonic impulses for a few seconds after the distention was released which was frequently of greater amplitude than the original clonus waves. This occurred while the pressure was falling, but stopped before the pressure reached 0 mm. of mercury.

The reflex was much more easily elicited and more sustained in some dogs than in others although it was present in all dogs that we examined. Since the reflex is abolished by unfavorable anesthetic conditions, we feel that this is a plausible explanation for the variation.

Since stimulation of the hypogastric nerves causes emission of semen into the urethra as well as closure of the smooth muscle around the urethra, it appears that the pressure head thereby developed would be easily sufficient to elicit the clonic reflex here described and it seems reasonable that this urethral clonus is at least one of the mechanisms by which semen is ejaculated from the urethra.

SUMMARY

Distention of the membranous urethra of the male dog to 15 mm. of mercury or more caused regular rhythmic contractions of the striated

muscle surrounding this structure, due to impulses carried along the internal pudic nerve. The clonus was easily elicited, long sustained, did not occur in female dogs and was abolished by moderate or deep ether anesthesia. The rate was relatively uniform about 3 contractile waves per each second of time.

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THE CHLORIDE CONCENTRATION OF GASTRIC SECRETION FROM FUNDIC POUCHES AND FROM THE INTACT WHOLE STOMACH

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During the course of experimental studies on the regulation of gastric acidity it was observed that the chloride concentration of the secretion from fundic pouches was practically constant under a variety of conditions while the chloride concentration of the secretion from the intact whole stomach showed marked variations. A review of the literature shows that recent workers have reported widely different values for the chloride concentration of the secretion from the intact whole stomach, the values varying from approximately 350 mgm. per 100 cc. to 590 mgm. per 100 cc. The present communication is an analysis of the factors responsible for these variations.

METHODS AND PROCEDURE. The general methods and calculations employed have been described in detail in previous papers (1933) (1934) and need not be repeated.

A. *Fundic pouch experiments.* The present analysis is based on 64 experiments performed upon five fundic pouches of the Heidenhain type. The conditions were purposely varied as much as possible. The pouches were stimulated either by histamin (synthetic) or by a meat meal in the main stomach. In some experiments the pure secretion was withdrawn from the pouch and analyzed while in others the pouch was filled with either distilled water, 0.1 normal hydrochloric acid or 0.173 normal hydrochloric acid containing phenol red.

We have previously shown (1933) that the chloride concentration of fundic secretion is practically the same in pure secretion as it is when the pouches are filled with the above mentioned solutions. The average value for 36 experiments was 578 mgm. of chloride per 100 cc. of secretion.

The present analysis deals particularly with the influence of the rate of secretion. It was found that the rate of secretion was much greater with histamin stimulation than with meat in the main stomach but that the chloride concentration of the secretion was approximately the same with

both types of stimulation. In figure 1 the 64 experiments have been grouped according to the actual amount of secretion by the pouch during the half-hour period. The amount of secretion in individual experiments varied from 2 to 31 cc. per half-hour. The average chloride concentration for all experiments was 594 mgm. per 100 cc., while most of the group averages range between 583 and 595 mgm. per 100 cc. In figure 1 the individual values for the chloride concentration in each group are shown. It is seen that the greatest spread occurs with the smallest amounts of

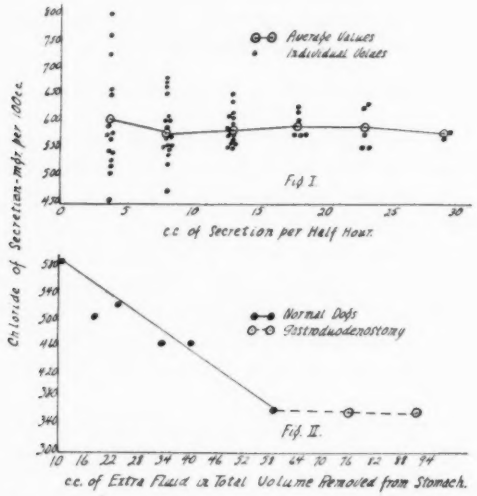
Average Values
Individual Values
Fig. I.
0 5 10 15 20 25 30
cc. of Secretion per Half Hour.
Chloride of Secretion-mgr. per 100 cc.
Normal Dogs
Gastroduodenostomy
Fig. II.
10 16 22 28 34 40 46 52 58 64 70 76 82 88 94
cc. of Extra Fluid in Total Volume Removed from Stomach.

Fig. 1 shows the chloride concentration of the secretion from fundic pouches with varying rates of secretion. An analysis of 64 experiments.

Fig. 2 shows the relationship between the chloride concentration of gastric secretion and the amount of extra fluid in experiments on the intact whole stomach. Solid line shows average values for 68 experiments performed upon four normal dogs. Dotted line shows values on a dog with gastroduodenostomy on which the same type of experiment was performed.

secretion and that the spread becomes progressively less as the amount of secretion becomes greater. This is probably due, for the most part, to the greater difficulty in determining the exact dilution of the phenol red when the dilution is very small. While the average chloride concentration is practically constant for all rates of secretion, the grouping of the individual values suggests that there is possibly a slight decrease in the chloride concentration when the secretion rate is low. This is probably due to the mucus which is secreted by the fundic region and which probably contains a lower concentration of chloride than the acid secretion. The

diluting effect of the mucus would be more marked with smaller amounts of acid secretion, although within the limits of secretion shown in figure 1 this effect is relatively insignificant and introduces variations which approach the limits of experimental error.

The results of the present studies and of those previously reported (1933) show that the chloride concentration of fundic secretion is practically constant at an average value ranging between 578 and 595 mgm. per 100 cc. and that the average value is practically unaltered by the concentration of acid in contact with the fundic mucosa or by the rate of secretion.

B. *Experiments on the intact whole stomach.* The present analysis is based on 68 experiments of the block type performed upon four normal dogs. From 300 to 400 cc. of a standard hydrochloric acid solution, containing phenol red, were introduced into the stomach. Histamin was used to stimulate gastric secretion. Three one-half hour experiments were performed after the injection of histamin.

The chloride concentration of the gastric secretion in these 68 experiments ranged from 353 to 669 mgm. per 100 cc. The distribution of the values between these two extremes was fairly uniform.

We have previously shown (1934) that the constancy of the chloride concentration of the fundic secretion makes it possible to separate the total fluid which enters the intact whole stomach into two fractions: first, the fluid of secreted hydrochloric acid which is not neutralized and second, the "extra fluid" which is composed of secreted hydrochloric acid which is neutralized, pyloric secretion and regurgitated duodenal secretions in varying proportions. By far the greater part of the extra fluid is composed of the two latter secretions. The amount of extra fluid of intragastric origin (pyloric) was found to be rather limited in amount, the total quantity for one-half hour periods averaging approximately 17 cc. Amounts definitely in excess of this value are probably of duodenal origin because of the parallel increase in bile pigments in the gastric contents. The chloride concentration of the pyloric and duodenal secretions obtained by acid stimulation were found to average 376 and 310 mgm. per 100 cc. respectively. Both of these values are definitely lower than the chloride concentration of fundic secretion.

When the 68 experiments under consideration were grouped according to the amount of "extra fluid" present in the gastric sample it was found that there was a direct relationship between the chloride concentration of the gastric secretion and the extra fluid, the former decreasing as the latter increased. In figure 2 this relationship has been emphasized by adding the values obtained on a dog in which a gastroduodenostomy had been performed and in which large amounts of extra fluid of duodenal origin entered the stomach. The average results in each of the four normal dogs showed the same relationship.

While the above analysis gives a partial explanation for the wide variations found in the chloride concentration of gastric secretion it fails to consider variations in the amount of acid secreted which must likewise have an important influence upon the chloride concentration of the combined gastric secretions. In figure 3 the relationship between the acid fluid, the extra fluid and the chloride concentration of the gastric secretion is shown. From this it is seen that the chloride concentration of gastric secretion is dependent upon two variable factors, namely, the amount of acid secreted which has a chloride concentration of approximately 578 mgm. per 100 cc. and the amount of extra fluid of pyloric and duodenal origin which has a chloride concentration varying between 376 and 310 mgm. per 100 cc. When the amount of acid secreted is very low the

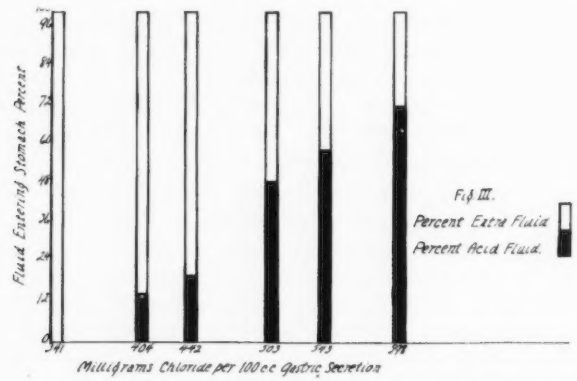

Fig. 3 shows the relationship between the chloride concentration of gastric secretion in the intact whole stomach and the per cent of acid and extra fluid. Curve constructed from the average results of 68 experiments on four normal dogs.

chloride concentration of the gastric secretion will approximate that of the extra fluid, while when the amount of acid secreted is very high the chloride concentration of gastric secretion will approach the value obtained in fundic pouches.

Discussion. The above analysis shows that the total fluid entering the stomach is normally composed of two and frequently of three secretions of widely different chloride concentration. The chloride concentration of the combined secretions will depend upon the relative proportions of each. When there is no bile present in the gastric contents, the chloride concentration will be low when small amounts of acid are secreted (approximately 340 mgm. per 100 cc.), but will increase as more acid is secreted. Bulger, Stroud and Heideman (1928) and Gorham, Stroud and Huffman

(1928) reported that the chloride concentration of gastric secretion in the fasting stomach approximated 360 mgm. per 100 cc. and that there was little change when acid secretion took place. In their experiments they removed and analyzed the fasting secretion and then placed 400 cc. of distilled water containing phenolsulphonphthalein in the stomach. The fluid was removed and analyzed at regular intervals. No stimulation other than the presence of the water in the stomach was given. The amount of acid secreted was usually very small and the chloride concentrations of the total secretion approximated 350 mgm. per 100 cc. In certain subjects, however, a greater secretion of acid occurred and as the acid secretion increased the chloride concentration of the mixed gastric secretion showed a parallel increase, the highest average value being 435 mgm. per 100 cc.

Berglund, Wohlquist and Sherwood (1927) and Polland, Roberts and Bloomfield (1928) stimulated the stomach with histamin and found that the chloride concentration of the gastric secretion varied between 525 and 600 mgm. per 100 cc. In these experiments the acid secretion greatly overshadowed the extra fluid of pyloric or duodenal origin.

Bloomfield, Roberts and Polland (1928) showed that the chloride concentration of gastric secretion in achlorhydria averaged 224 mgm. per 100 cc. after the injection of histamin and that all values were below 440 mgm.

SUMMARY

1. The total fluid entering the stomach is normally composed of two and at times of three separate secretions, the chloride concentrations of which are widely different. Fundic secretion (acid + mucus) averages approximately 578 mgm. of chloride per 100 cc. and this value is practically uninfluenced by the rate of secretion or the hydrogen ion concentration of the gastric contents. Pyloric and mixed duodenal secretions have a much lower chloride concentration, the average value approximating 340 mgm. per 100 cc.

2. The chloride concentration of mixed gastric secretions can vary from approximately 340 to 595 mgm. per 100 cc. depending upon the relative amounts of the various separate secretions. When little or no acid is secreted the lower figures will be found, but the value will rise as more and more acid is secreted, unless a large amount of duodenal fluid enters the stomach in which case the value may be low in spite of considerable secretion of acid.

3. The above considerations explain the widely different values for the chloride concentration of gastric secretion found in the literature and show that experiments on the intact whole stomach cannot be used to prove that the chloride concentration of the acid secreted by the stomach varies with the rate of acid secretion.

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METABOLIC RATE, BLOOD SUGAR AND THE UTILIZATION OF CARBOHYDRATE

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The many observations on blood sugar in exercise have revealed widely different conditions. Thus one may have extreme hypoglycemia as found by Levine, Burgess and Derick (1) in some marathon runners, or at the other extreme, hyperglycemia as in football players (2). A moderate decrease in the concentration of sugar in the blood (B. S.) has been found by Christiansen (3) in exercise of one hour's duration; in recovery following exercise both Rakestraw (4) and Christiansen report moderate increases. We have found that when exercise is carried on for many hours without emotional stress the value of B. S. may not vary much from the resting level until a terminal hypoglycemia develops (5). It seems probable that such a state reflects the exhaustion of glycogen reserves; if so it should be associated with a decreasing respiratory quotient.

With this possibility in mind we have undertaken a series of experiments designed to study the relation between metabolic rate (M. R.), respiratory quotient (R. Q.), carbohydrate reserve,[1] and blood sugar (B. S.). The experimental procedure has been described in part elsewhere (6). In these experiments two rates of work were employed with alternation of rate each half-hour. The work was carried on for several hours, *viz.*, long enough to reduce materially the reserve of carbohydrate. During each half-hour blood was obtained from the finger while work proceeded and sugar was determined by the Folin-Malmros method (7). At the same time expired air was collected and analyzed in the usual manner. Since the rate of work did not exceed the oxygen transport capacity the rate of oxygen intake is a measure of the M. R.

Our ablest subject was Y, aged 19, a skilled runner who has finished near the front in marathons. In three experiments he ran for 6 hours alternating at rates of 9.3 and 11.3 km. per hour with an energy output on these occasions averaging 3400 Cals. The second of these experiments

[1] While the carbohydrate reserve cannot be measured directly, changes in it can be calculated from the M. R. and R. Q. In our experiments on subject Y the amount of carbohydrate used exceeded the usual estimates of glycogen content of man thus indicating that carbohydrate reserve was reduced to a low level.

TABLE 1

Subject Y—January 7, 1933

Room temperature 14°; body temperature in work 38.8 ± 0.2; urine 340 cc.; urine N., 2.5 grams; energy from protein, 2 per cent; duration of each work period 25 minutes; of each intervening rest period, 5 minutes.

OXYGEN USED	R.Q.	BLOOD SUGAR	BODY WEIGHT	ENERGY PRODUCED	SOURCES OF ENERGY			SUMMATION OF CARBOHYDRATE USED
					Carbohydrate		Fat	
l. per min.		*mgm. per 100 cc.*	*kgm.*	*cal.*	*cal.*	*per cent*	*cal.*	*gm.*
		100	59.61					
2.46	0.97	82	59.32	308	276	90	27	65
2.10	0.96	89	59.03	262	225	85	32	118
2.43	0.94	87	58.82	302	238	79	59	174
2.07	0.88	79	58.65	254	148	59	101	209
2.47	0.86	83	58.25	301	155	51	141	246
2.15	0.82	81	58.15	260	99	38	156	270
2.55	0.82	75	57.93	308	118	38	185	298
2.19	0.79	78	57.78	262	74	28	183	316
2.55	0.82	69	57.48	308	118	38	185	344
2.23	0.79	74	57.30	267	75	28	187	362
2.58	0.81	66	57.55	311	108	35	198	388
2.22	0.77	58	57.35	264	56	21	203	401
				3407	1690	50	1657	

TABLE 2

Summary of 3 experiments on subject Y

Energy derived from protein assumed to be 5 cals. per period; duration of each period, twenty-five minutes

PERIOD	OXYGEN USED	R.Q.	BLOOD SUGAR	ENERGY FROM CARBOHYDRATE	Σ CARBOHYDRATE
	l. per min.		*mgm. per 100 cc.*	*per cent*	*gm.*
1	2.57	0.93	90	76	58
2	2.21	0.92	101	72	105
3	2.56	0.91	82	69	156
4	2.11	0.88	82	59	192
5	2.56	0.88	82	59	236
6	2.15	0.83	80	42	262
7	2.50	0.82	82	39	289
8	2.18	0.78	85	25	304
9	2.55	0.81	76	35	330
10	2.19	0.77	80	21	343
11	2.54	0.79	67	28	363
12	2.17	0.76	63	18	374

is presented for an example in table 1 and a summary of the three experiments on him in table 2. Subject H was in good physical condition, but not a skilled runner. Beginning after a light breakfast, he worked for 7 hours on 4 occasions with glucose ingestion in the latter part of 2 experiments. The detailed experimental results on him are omitted; they are presented in condensed fashion in figures 1 to 3 and table 3. Two other subjects, B and C, were used in single experiments only. Their response was similar; the results on B. S., R. Q. and M. R. are included in figure 2 and table 3.

Metabolic rate and blood sugar. The relation of B. S. to M. R. has been considered recently by Christiansen (3). Accordingly it will be of interest to see how our results agree with his conclusion that there is no relation

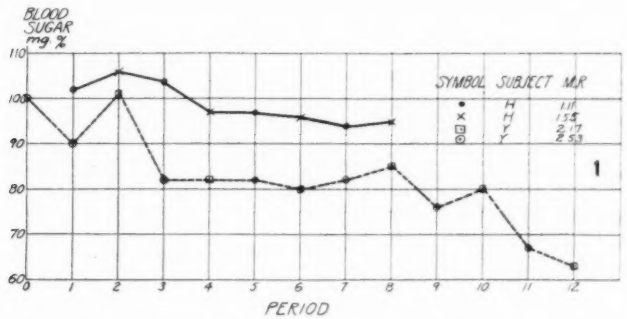

Fig. 1. Blood sugar during exercise. Four experiments on subject H are averaged, 3 on subject Y. H used 163 grams of carbohydrate in 8 periods (4 hours) and Y used 374 grams in 12 periods (6 hours). Virtually all carbohydrate was derived from reserve glycogen.

between these variables. The results of 9 experiments on 4 subjects including 118 pairs of observations are summarized in table 3.

These results include a number of observations on subject H, after glucose ingestion. In consequence the mean B. S. in his case is much higher than that of subject Y. Apart from this, however, the two subjects showed a significant individual difference throughout. This is evident from figure 1 which gives B. S. as a function of time in the two subjects, excluding observations made on subject H after glucose administration. From the trend of these curves and from the summary in table 3 we may conclude that there is not an intimate relation between M. R. and B. S. under the conditions of our experiments. Furthermore it appears that 4 hours of hard work may be carried on before marked hypoglycemia develops.

Thus these experiments support Christiansen's conclusion that B. S.

does not depend on M. R. It seems that, so long as exercise is continuous and reserves of carbohydrate ample, B. S. is maintained near the resting

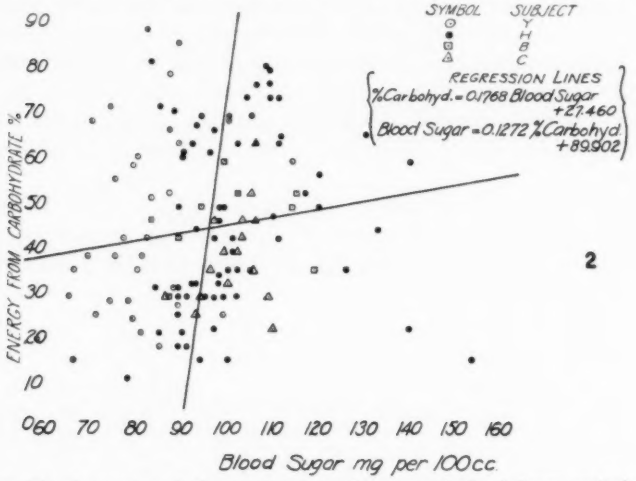

Fig. 2. Blood sugar and the proportion of energy derived from carbohydrate. Results after glucose ingestion are included.

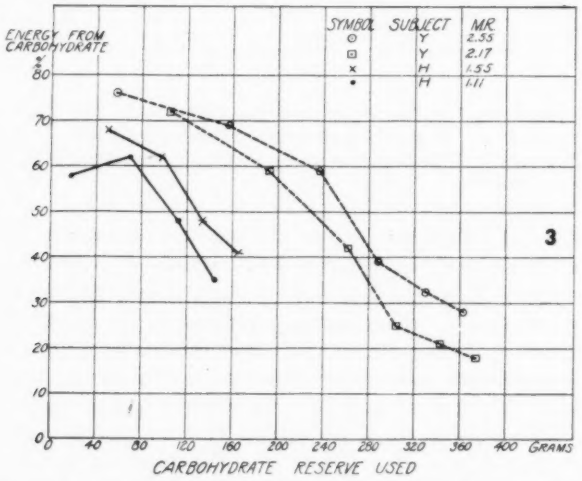

Fig. 3. The reserve of carbohydrate and the proportion of energy derived from carbohydrate. The experiments are averaged as in figure 1.

level by increasing glycogenolysis to a rate commensurate with the rate of carbohydrate oxidation. The data of Christiansen and unpublished ob-

servations of our own commonly show an increase in B. S. when vigorous exercise is interrupted; the more intense the exercise the more abrupt is the increase. This hyperglycemia may mean that glycogenolysis returns to normal more slowly than the rate of glucose utilization. In consequence of this phenomenon and the fact that most games are severe and discontinuous in character it is probably true that during them the B. S. reaches and persists at high levels. This certainly is true in football playing—a game which lasts for 2 hours with only about 8 minutes of active play (2).

Utilization of carbohydrate and blood sugar. We have seen that in muscular activity at two levels the B. S. differs but little from its value in rest. From this one might guess that the proportion of carbohydrate used also remains constant. To test this a study has been made of the relation between B. S. and proportion of energy derived from carbohydrate

TABLE 3

Blood sugar in relation to metabolic rate

SUBJECT	NUMBER OF OBSERVATIONS	MEAN OXYGEN INTAKE	MEAN BLOOD SUGAR
		l. per min.	*mgm. per 100 cc.*
H	31	1.13	104
H	28	1.57	102
Y	18	2.17	82
Y	17	2.54	80
B	4	1.62	103
B	4	2.30	101
C	8	1.26	103
C	8	1.69	99

as determined by the R. Q. Figure 2 contains 125 pairs of observations on the 4 subjects. Obviously there is no more than a slight interdependence of these two variables under the conditions of our experiments. The correlation coefficient, calculated by conventional methods, comes out $+0.150 \pm 0.59$.[2] The fact that it is positive is probably associated with

[2] In more recent and unpublished experiments we find a much higher correlation between these variables. The experimental conditions, however, are significantly different. The rate of work is constant, not variable; rest periods of one hour or more are interposed between working periods of two hours; the subject begins in a post-absorptive state and is given no food during the entire experiment. We are not able to say which difference is most significant but it is probable that the last point mentioned is important. It was observed, for example, that after administering glucose to subject H the R. Q. did not reach a maximum for two hours, i.e., some time after the blood sugar had passed through its maximum. This phenomenon will be referred to below; it means that the administration of sugar during exercise disturbs the existing relation between blood sugar and proportion of carbohydrate used.

the facts that at the beginning of exercise both variables are high and at the end both are low. In the intervening hours the B. S. fluctuates irregularly while the R. Q. decreases with a fair degree of uniformity.

Utilization of carbohydrate, carbohydrate reserve and metabolic rate. We show in figure 3 the relation between these variables in subjects H and Y. On the basis of this figure and of additional observations of the R. Q. in rest it is evident that the ratio of carbohydrate burned to fat burned increases with metabolic rate. We cannot yet describe the relation precisely. From studies now in progress it appears that the proportion of carbohydrate utilized increases slowly with the first increments of M. R. and more rapidly as the capacity for oxygen transport is approached. It is also evident from figure 3 that even early in exercise when reserve of carbohydrate is greatest the utilization of carbohydrate is significantly less than 100 per cent even when the M. R. is 12 times its resting value. Fat continues to be burned even with a plethora of carbohydrate.

From this evidence we can not conclude that carbohydrate is essential for strenuous exercise. While our subjects were nearly exhausted it does not follow that the principal source of their fatigue was carbohydrate depletion. Thus we have shown elsewhere that the M. R. of man may be 700 per cent of the resting level for 2 hours with less than 10 per cent of the energy derived from carbohydrate without excessive fatigue. And in the dog a much higher M. R. is possible for as long as 27 hours without fuel. Possibly in man the accumulation of acetone bodies in exercise of long duration without food is a primary source of breakdown; this question is now under investigation.

The intimate relation between the proportion of energy derived from carbohydrate and reserve of carbohydrate is particularly interesting in view of the apparent lack of close relation between B. S. and carbohydrate utilization. Although the choice of fuel in exercise is determined with precision, its governor is at present unknown.

Utilization of carbohydrate after glucose ingestion The experiments of Carpenter and Lee (8) have already shown that the increase in utilization of glucose begins within 30 minutes after glucose ingestion and reaches a maximum within 60 minutes after ingestion. In one of our experiments on subject 4 the peak in the blood sugar curve coincides with the lowest value of R. Q. observed in that experiment. In the other experiment the maximum R. Q. comes two hours after glucose ingestion when B. S. has returned to its pre-ingestion value. The apparent lag in response of the R. Q. is all the more remarkable because of the depletion of glycogen reserve by the previous activity. It suggests that the utilization of glucose by the muscles takes second place to its use for replenishing glycogen stores. It is possibly significant in this connection that Carpenter

and Lee (8) have found a more rapid and greater increase in R. Q. after ingestion of fructose or galactose than after glucose.

SUMMARY

A study has been made of exercise of sufficient duration and intensity to reduce the carbohydrate reserve to low levels. Altering the rate of work each half-hour alters the proportion of energy derived from carbohydrate (measured by the R. Q.) but not the blood sugar. With a plethora of carbohydrate some fat is used in exercise and as carbohydrate reserve diminishes the proportion of energy derived from fat may increase from 8 per cent to 77 per cent (table 1). There is no evidence that carbohydrate is more essential in work than in rest.

Under the conditions of our experiments there is a small positive correlation between blood sugar and R. Q. While the choice of fuel is determined with precision, blood sugar concentration is not the governor. Thus, when glucose is ingested during work and after depletion of carbohydrate reserves, the blood sugar may reach a maximum before there is a response of the R. Q.

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THE EFFECT OF THE VAGUS NERVES ON SUGAR TOLERANCE IN DOGS[1]

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This problem was undertaken in an attempt to come to some definite conclusion regarding the influence of the vagi nerves on the secretion of the islands of Langerhans. The literature is contradictory.

Clark (1) has apparently demonstrated that the pancreas is responsible for changes in blood sugar level after vagotomy—having eliminated the suprarenals and liver from consideration as factors. He has consistently maintained (1), (2), (3), that right vagus section in the neck, or above or below the diaphragm (in unanesthetized animals and in animals under various types of anesthesia) results in a slight fall in blood sugar immediately and an increased sugar tolerance for some weeks which is later followed by a diminished tolerance.

Quigley, Halloran and Barnes (4) did not concern themselves with the acute changes which might follow section of the vagi (made just above the diaphragm) but used animals that had been bilaterally vagotomized from one to six months before, in whom adaptive changes would be well stabilized. In their series of experiments there was an earlier return to the original level in the vagotomized animals after administration of glucose and the fall below the original level was more marked than in normal animals.

Hoet and Ernould (5), on the other hand, using rabbits anesthetized with Numal report decreased sugar tolerance after double vagotomy.

Numerous other investigators (6) have stimulated the vagi and noted a subsequent hypoglycemia. This group concludes that the vagi carry secretory fibers to the islands of Langerhans.

In an attempt to clear up the situation, Friedenwald et al. (7) ran a series of experiments on dogs and were unable to find an increased sugar tolerance for the first few weeks after vagus section followed by diminished tolerance. They state that minor changes following vagus section disappeared quickly and that the animals after three months responded normally in every way.

[1] Aided by the Biological Research Fund of the Rockefeller Foundation.

Thus at the present time no unanimity of opinion exists as to the function of the vagus fibers to the islands of Langerhans.

TECHNIQUE. The dogs used in this series were, with the exception of the first, females who had been trained to lie quietly for three or four hours. Blood sugar determinations were made, using the Shaffer-Hartman sodium tungstate precipitation method. The dogs were fasted for 30 to 36 hours before the glucose injection (1 gram per kilo body weight in 100 cc. 0.9 per cent NaCl) and blood samples were taken before and at fifteen minute intervals after administration. The first four experiments were run on dogs whose normal tolerance had been determined and on whom subsequent bilateral vagotomy just above the diaphragm had been performed. In all cases a piece of nerve 2 to 3 cm. in length was excised in order to prevent subsequent approximation of the cut ends. Ether anesthesia was used during the operations.

After this series, the question as to whether the operation itself (without vagotomy) altered the sugar tolerance curve presented itself. To answer this the last three dogs were subjected to a "dummy" operation, after normal tolerance tests had been run, and before bilateral vagotomy. The procedure in the "dummy" operation consisted in pulling the esophagus to the opening made in the chest wall, and, with care taken not to injure the vagi nerves, handle it as would be done in an operation for vagotomy.

RESULTS. Table 1 is a compilation of results received in all seven experiments. The column on the right, headed "recovery time in minutes," indicates the length of time required for the blood sugar to return to the normal level after the intravenous administration of glucose.

The blood sugar of dog 1 normally returned to the original level in 135 minutes whereas after vagotomy a decrease in tolerance set in almost immediately. In about two and a half weeks the tolerance returned to normal and the blood sugar, which showed a marked drop after vagotomy, also regained the original level.

The same general curve is illustrated in the case of dog 2—the tolerance test after vagotomy approaching normal in three weeks although the blood sugar level has not yet reached the former mark. It is interesting to note in this case that a tolerance test run one day after vagotomy shows little or no difference from those run before vagotomy.

The third and fourth experiments of this series tend only to confirm those preceding. The decreased tolerance, fall in normal blood sugar level and the subsequent return to normal of both is essentially the same.

Comparison of normal sugar tolerance tests with those after a "dummy" operation and then with those made after bilateral vagotomy on the same dog was attempted in the last three experiments. As seen in table 1, all three dogs (nos. 5, 6 and 7) showed a decrease in tolerance and a drop in blood sugar level after the "dummy" operation. Subsequent vagotomy

TABLE 1

Unanesthetized dogs. Blood sugar before and after intravenous injection of 1 gram of glucose per kilo, blood sugar samples at 15 minute intervals

		NUMBER OF DAYS AFTER OPERATION	NORMAL BLOOD SUGAR	BLOOD SUGAR AFTER INTRAVENOUS INJECTION													RECOVERY TIME
																	minutes
Dog 1	A*		108	107	212	183		168			137		110					135
			82	83	200	174		168			113		85					135
	B	3		70	248	200					174		149		123		96	210+
		6	75	75	242	214		176					110		101		89	210+
		17	80	82	236	194		140			96		78	67			62	135
Dog 2	A			102	243	160	107	100	95	84								45–60
				105	209	145	111	98	94									45–60
	B	1		95	242	152	104	86	85	78								45–60
		2		84	265	220	149	154	135	114	93							105+
		7		88	271	225	197	162	125	116	101	88						120
		18		85	231	157	127	103	94	84								90
Dog 3	A		94	94	213	137	104	93			90							60
				89	241	202	192	89		75								60
				88	249	163	110	85		78								60
				83	249	120	88	78		86								45–60
	B	1		89	232	151	127	103	95	89	91	92						90
		8		82	212	155	118	96	82	82	76	78						75
		12		77	257	152	105	83	72	74	76							60–75
		23		89	256	124	87	82	84									45
Dog 4	A			86	268	194	147	130	90	82								75–90
				92	254	213	168	130	97	84								75–90
				91	295	228	204	127	103	81								75–90
	B	1		78	202	188	174		128	109	99	100	98					135+
		2		66	210		161	140	119	106	100	88	82	82				150+
		5		63	260		222		174		136		117		96		90	195+
		12		87	227			138	96	85	75		67		76		74	90
		14		85	264	222	182	135	102	81	76							<90
Dog 5	A			86	211	145	100	77	80	81	83							45–60
				80	210	106	81	71		76								45
	C	2		79	207	183	159	115	107	94								90+
		5		77	206	162		89	82	75								90

* A—normal sugar tolerance; B—after double vagotomy; C—after "dummy" operation.

TABLE 1—*Concluded*

		NUMBER OF DAYS AFTER OPERATION	NORMAL BLOOD SUGAR	BLOOD SUGAR AFTER INTRAVENOUS INJECTION												RECOVERY TIME
																minutes
Dog 6	A		88	230	158	101	80	72	78							45–60
			89	218	143	98	83	77	71							45–60
	C	2	80	223	152	101	92	84	78							75–90
		4	81	217	149	105	94	87	79							75–90
	B	2	81	233	175	136	114	97	83	77	72					90–105
		29	79	218	146	92	69	74	70							45–60
Dog 7	A		103	306	168	116	92	99	102							45–60
			109	298	169	107	87	103	103							45
	C	2	96	311	242	210	166	141	115	93						105
		9	101	271	202	135	125	108	78							75–90
		11	98	246	182	120	102	81	93							60–75
	B	5	96	250	183	134	100	93	81	76	85					60–75
		7	102	259	180	145	115	97		77						60–75

caused only slight additional change in the sugar tolerance curve. The blood sugar level remained unchanged.

Dog 6 developed a severe stomach disorder three days after vagotomy. This condition lasted for three weeks—during which time she vomited repeatedly—though fed no solid food. Glucose and bismuth subnitrate were administered by stomach tube. Because of this only one sugar tolerance test was run immediately after vagotomy. Although this test showed a variation from the normal, it was quite similar to those taken after the "dummy" operation. One month later a sugar tolerance test indicated that the dog had returned to normal with the exception of the blood sugar, which remained at a low level.

The sugar tolerance of dog 7 after the "dummy" operation was less than that following vagotomy but the blood sugar level remained the same in both cases, slightly under normal.

SUMMARY

The results of the first four experiments—without consideration of the later tests in which the "dummy" operation was introduced—leads to the conclusion that bilateral vagotomy above the diaphragm causes a temporary fall in the normal blood sugar level and a temporary decrease of the sugar tolerance. The peak of the decreased sugar tolerance occurs from 3 to 5 days after vagotomy. The return to normal occurs gradually in two to three weeks.

But since the "dummy" operation, which leaves the vagi intact, produces

the same change in blood sugar level and sugar tolerance as double vagotomy, it seems clear that the temporary changes in blood sugar and sugar tolerance after section of the vagi nerves are not due to elimination of insulin secretory fibers in the vagi nerves.

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THE EFFECT OF ADDITION OF MINERALS AND SUCROSE TO MILK AS SHOWN BY GROWTH, FERTILITY AND LACTATION OF THE RAT

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Hart, Steenbock, Waddell, and Elvehjem (1) showed that milk is deficient in copper and that this element plays a vital rôle in the synthesis of hemoglobin. Keil and Nelson (2) have recently made an extensive study of the rôle of copper and various other elements and amino acids in the regeneration of hemoglobin. They conclude that copper is specific in its effect on hemoglobin. Daniels and Hutton (3) found that rats failed to reproduce on milk with added Fe, but reproduction did occur when a mineral mixture of NaI, NaF, $KAl(SO_4)_2 \cdot 12H_2O$, $MnSO_4 \cdot 4H_2O$, and Na_2SiO_3 was added. They state that all of these salts are necessary in the milk for normal reproduction. This work was done before the significance of Cu was recognized. It is certain that animals can not grow and reproduce without Cu, consequently there may have been contamination of this element in the milk, starch, or salt mixture used by Daniels and Hutton. It is also very likely that coprophagy was an additional source of this element. Since there must have been contamination of Cu somewhere in the course of the experiments by Daniels and Hutton, the question naturally arises as to whether the results of these investigators were due to the added Cu or whether the other elements played a significant rôle in reproduction.

EXPERIMENTAL. All of the experiments were performed on rats. The milk used for these studies was collected directly into glass containers from pure bred Holstein cows, in order to avoid contamination with copper and other elements. C. P. inorganic salts were recrystallized and examined by a Hilger quartz prism spectograph, in order to insure their purity. The animals were placed on galvanized screens to eliminate coprophagy.

Chart 1 shows that female rats on milk plus $FeCl_3$ and $CuSO_4 \cdot 5H_2O$ grew at a rate considerably slower than rats on our stock ration. The addition of Na_2SiO_3 to the above ration did not improve the rate of growth. On the other hand substitution of $KAl(SO_4)_2 \cdot 12H_2O$ for the silicate gave results on growth which were better than those obtained with the other two rations. The addition of NaF to the milk supplemented with $FeCl_3$

and $CuSO_4 \cdot 5H_2O$ gave results which surpassed those on $KAl(SO_4)_2 \cdot 12H_2O$ for the first four months; later the fluoride exhibited toxicity, mottled enamel developed, and the rats declined in weight. At six months one of these animals succumbed and the remainder appeared in a very poor physical state, so that the NaF was eliminated and a mixture of $KAl(SO_4)_2 \cdot 12H_2O$, Na_2SiO_3, and $MnSO_4 \cdot 4H_2O$ was added. The improvement in appearance was remarkable, and the curves of growth immediately started upward. Rats on milk, $FeCl_3$, $CuSO_4 \cdot 5H_2O$, and the Daniels and Hutton salt mixture (3), exclusive of iodine, grew satisfactorily and performed about equally well as rats on our stock ration. Rats on milk, $CuSO_4 \cdot 5H_2O$,

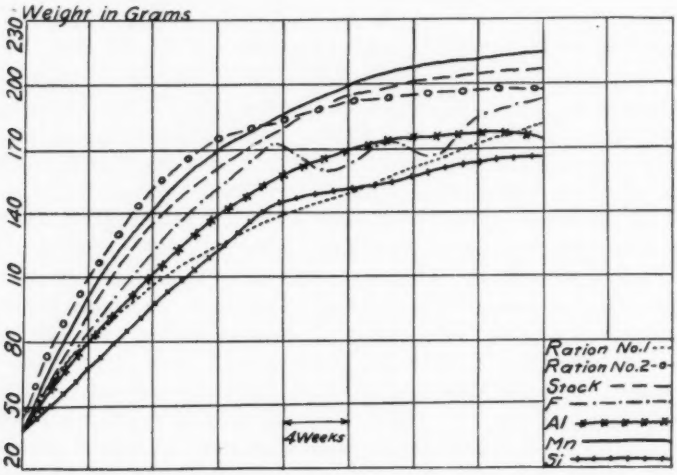

Chart 1. Growth curves of females. Ration 1 consists of milk ad lib., Cu as $CuSO_4 \cdot 5H_2O$, 0.05 mgm. daily, and Fe as $FeCl_3$ 0.50 mgm. daily. Ration 2 consists of ration 1 plus the Daniels and Hutton salts exclusive of iodine. The salts of F, Al, Mn, and Si were added to ration 1 at the rate of 1.5 mgm. each daily. Six females were used on each ration.

$FeCl_3$, and $MnSO_4 \cdot 4H_2O$ performed better than any of the previous lots and even surpassed those on the stock ration. Previously Nelson, Evvard, and Sewell (4) had shown that Mn in certain synthetic diets stimulated the growth of rats. $MnSO_4 \cdot 4H_2O$, $KAl(SO_4)_2 \cdot 12H_2O$, NaF, and Na_2SiO_3 were administered orally to the extent of 1.50 mgm. each daily. The curves are composite and include the females only. Eight animals, two males and six females, were employed in each lot.

Table 1 (expts. 1 to 6 inclusive) gives results on reproduction and lactation for three generations on milk, $CuSO_4 \cdot 5H_2O$, $FeCl_3$, and the Daniels and Hutton salt mixture, exclusive of iodine, and a comparison with results

obtained on milk, $CuSO_4 \cdot 5H_2O$, and $FeCl_3$ for two generations. It will be observed that the former animals reproduced very well, although possibly not so much so as on our stock ration; and that the mortality of the young on the former ration was very low. However, reproduction became poorer in succeeding generations. The rats on milk, Cu, and Fe in the first generation reproduced equally well with the animals on the Daniels

TABLE 1

Reproduction and lactation records

EXPERIMENT NUMBER	SUPPLEMENT	NUMBER OF FEMALES USED	TOTAL LITTERS BORN	NUMBER YOUNG BORN	AVERAGE WEIGHT OF YOUNG AT BIRTH	NUMBER YOUNG ATTEMPTED TO WEAN	NUMBER YOUNG WEANED	MORTALITY OF YOUNG	AVERAGE WEIGHT OF YOUNG AT 30 DAYS
								per cent	
1 F	D.H. less NaI	7	26	154	5.0	65	63	3.0	48.1
2 S	D.H. less NaI	4	9	65	5.1	24	20	16.6	47.0
3 T	D.H. less NaI	5	5	35	5.1	35	35	0.0	38.5
4 F	—	7	24	161	4.3	75	34	54.6	41.1
5 S	—	9	0	0	0.0	0	0	0.0	0.0
6 S	D.H. less NaI	9	14	76	4.6	28	27	3.5	55.2
7 S	Si	5	0	0	0.0	0	0	0.0	0.0
8 S	F	5	0	0	0.0	0	0	0.0	0.0
9 S	Al	5	0	0	0.0	0	0	0.0	0.0
10 S	Mn	4	9	63	5.1	56	55	1.7	53.3
11 +	Mn + Al	4	11	60	4.7	50	32	36.0	49.3
12 +	Mn + Si	4	9	37	4.9	37	17	54.0	45.9
13 +	Si + Al	5	0	0	0.0	0	0	0.0	0.0
14 Fo	D.H.	4	4	22	5.7	22	20	9.0	52.1
15 Fi	D.H.	7	2	15	4.7	15	7	53.3	45.8
16 F	Sucrose	7	10	56	4.5	56	22	60.7	38.0

The letters following the experiment number designate the generation. F denotes first generation, S second generation, T third generation, Fo fourth generation, and Fi fifth generation. + Second generation animals from females on milk, Cu, and Fe. The diets of all animals aside from the supplement consisted of milk ad lib., and daily 0.05 mgm. Cu as $CuSO_4 \cdot 5H_2O$, and 0.50 mgm. Fe as $FeCl_3$. The symbols Si, F, Al, and Mn refer to Na_2SiO_3, NaF, $KAl(SO_4)_2 \cdot 12H_2O$ and $MnSO_4 \cdot 4H_2O$. These salts were administered to the extent of 1.5 mgm. each daily.

and Hutton salt mixture, but the mortality of the young was considerably greater, and the weight of the young at birth and weaning time was less than on the ration containing the complex salt mixture. The male and female rats of the first generation on milk, Cu, and Fe proved fertile for at least 24 months. However, rats on this same diet do not reproduce in the second generation. The nine females (expt. 5) of the second generation on milk, Cu, and Fe failed to produce a single litter in eight months; but

with the addition of the Daniels and Hutton salt mixture seven had young (expt. 6). The two that did not reproduce had lung infection and died. Infection of the middle ear, otitis media, was very prevalent in the second generation rats receiving milk, Fe, and Cu; it was not nearly so common in the first generation. Seven animals of 32 first generation rats on milk, Cu, and Fe had otitis media, whereas 35 of the 73 animals of the second generation showed this pathological condition. Rats on rations without Mn were especially prone to otitis media. Two of the 88 animals on Mn exhibited this condition, whereas 63 of the 141 animals not receiving Mn were infected. The animals recorded in table 1 (expts. 1 to 6 inclusive) were on experiment for one year. It is evident from the data that something else is required besides Cu and Fe, if animals on milk are to continue reproduction. It is also apparent that the addition of the Daniels and Hutton salt mixture markedly improves reproduction in rats receiving milk, Cu, and Fe. The next problem was to determine why animals on milk, Cu, and Fe fail to reproduce in the second generation. Since the Daniels and Hutton salt mixture proved beneficial the question naturally arose as to whether all the elements in this salt mixture were necessary. The amount of Cu used in these experiments was 0.05 mgm. Cu as $CuSO_4 \cdot 5H_2O$ daily. The concentration of Fe employed was 0.50 mgm. Fe as $FeCl_3$ daily. The other salts were added to the extent of 1.50 mgm. each daily.

Data in table 1 (expts. 7 to 10 inclusive) show the effects produced in the second generation rats on a ration of milk, Cu, and Fe supplemented with the four different salts indicated. Reproduction and lactation on these same diets in the first generation were about the same as in the first generation on milk, Cu, and Fe, but the Mn addition proved better for reproduction and lactation, and Al gave poorer results for lactation than obtained on the milk, Cu, and Fe ration. These animals were on experiment for nine months. The concentrations of salts used in these experiments were the same as for those given in experiment 1. Experiments 7 to 10 indicate that Mn plays a different rôle than Al, F, and Si, for by the addition of this element to a diet of milk, Cu, and Fe sterility was averted in the second generation. Further evidence of the importance of Mn in reproduction is shown in table 1 (expts. 11, 12 and 13).

Table 1 (expts. 11, 12 and 13) shows the effect of supplementing a milk, Cu, Fe ration with two salts. The rats employed in this experiment were second generation animals on a ration of milk, Cu, and Fe. Second generation animals on milk, Cu, and Fe do not reproduce. The addition of Si and Al did not stimulate reproduction in these second generation rats on milk, Cu, and Fe; whereas, the addition of Mn did stimulate reproduction. Evidently the milk was deficient in Mn, and the results show that this element plays some rôle in reproduction. The animals were on

experiment for nine months. The salts and the amounts used are the same as given previously (expts. 7, 8, 9, and 10). The animals receiving Mn did not reproduce in the following generation, however; for the young were kept on these diets for at least ten months without the birth of a single litter. Since the young from the above females did not reproduce, it was thought that possibly fluorine and iodine present in the Daniels and Hutton salt mixture might be essential. Consequently the third generation animals, whose reproductive record is given in table 1 (expt. 3), were bred; and the results of the fourth and fifth generation are given in table 1 (expts. 14 and 15). The first three generations received the Daniels and Hutton salt mixture, exclusive of iodine; but iodine as NaI to the extent of 0.213 mgm. daily was included in the salt mixture after that. The fourth and fifth generation animals were on experiment for nine months.

It is evident from experiments 14 and 15 that reproduction is not normal in the fourth and fifth generation animals receiving milk, Cu, Fe, and the complete Daniels and Hutton salt mixture. At the conclusion of the experiment the animals were given growing ration, but not a single litter was born after the change. It is possible that some of the elements in this salt mixture are toxic. Our experiments are incomplete on this point. However, various combinations of the different elements in the Daniels and Hutton salt mixture have been added to milk, Cu, and Fe, but so far the results have been unsatisfactory. Reproduction may be stimulated for a short time, but inevitably failure ensues. It is possible that the failure of a ration consisting of milk, Cu, and Fe may not lie in a deficiency of inorganic elements but rather in organic substances such as protein and carbohydrate. Skinner, Van Donk, and Steenbock (5) say that for normal oestrum the prime deficiency of a milk-iron-copper-ration is energy. Their data show that in the case of the milk-sucrose-manganese group of animals oestrum had not been observed prior to the addition of this supplement, but after the addition it occurred once every 4.3 days. Animals receiving sucrose but no manganese exhibited somewhat longer, though regular, cycles of 5.4 days' duration. Prior to this work of the above investigators we had already performed experiments on the effect of the addition of sucrose to a milk, Cu, and Fe ration on reproduction and lactation. Table 1 (expt. 16) shows the effect of the addition of 5.0 grams of sucrose to 100 cc. of milk supplemented with Fe and Cu.

If the data in experiments 4 and 16 be compared, it is readily seen that the addition of sugar to the milk-Cu-Fe ration was detrimental to reproduction and lactation. Furthermore, the second generation animals which had received sugar failed to reproduce. The animals in experiment 16 were kept on this diet for 12 months.

SUMMARY

First generation animals on milk, Cu, and Fe have reproduced for as long as two years. Second generation rats on this diet do not reproduce, but the addition of the Daniels and Hutton salt mixture has a favorable effect on reproduction of the second generation on milk, Cu, and Fe.

The addition of $MnSO_4 \cdot 4H_2O$ to a diet of milk, Cu, and Fe makes reproduction possible through the second generation.

The addition of Mn and Si or Mn and Al to a diet of milk, Cu, and Fe so alters its nutritive value, that reproduction occurs in the second generation.

Although the Daniels and Hutton salt mixture improves reproduction when added to a milk, Fe, and Cu diet, nevertheless, reproduction is not normal especially in succeeding generations. The fifth generation on this diet proved sterile.

The addition of sucrose to a milk, Fe, and Cu ration gave results that were poorer than on milk, Fe, and Cu alone.

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THE RENAL EXCRETION OF SUCROSE, XYLOSE, UREA AND INORGANIC SULPHATES IN NORMAL MAN: COMPARISON OF SIMULTANEOUS CLEARANCES

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A comparison of the clearance of sucrose and xylose in normal individuals is of interest from several standpoints. One of these is the fact that conflicting data have been presented on the relative excretion of the two sugars. Jolliffe, Shannon and Smith have expressed the belief that the clearances are practically identical, but on the other hand, White and Monaghan found that the xylose clearance in dogs is definitely less than that of sucrose. For further comparison we have included also in our present studies estimations of both the urea and inorganic sulphate clearances. The sulphate clearance is thus for the first time simultaneously determined with that of the sugars, sucrose and xylose.

METHODS. *Physiologic procedure.* Five experiments were carried out on five normal individuals during the months of July, August and September, 1933. In each of the experiments clearances were obtained during two consecutive periods in the same morning. Each period afforded a control on the other, and in addition gave us a total of ten periods with simultaneous clearances of the four substances. No breakfast was taken, although 50 grams of xylose were ingested early in the morning with oatmeal porridge, the latter was suggested by Chasis, Jolliffe and Smith, to prevent diarrhea. This symptom developed in only two cases, and in these was moderate in degree. Approximately two hours later a 20 per cent solution of sucrose was injected intravenously. During the periods of collection the subject carried on light work. The details of a single experiment (B, table 1) were as follows:

7:00 a.m. Experiment begun. Emptied bladder. Ingestion of 50 grams xylose begun in 2 teacups of cooked oatmeal and 300 cc. water
7:30 a.m. Ingestion of xylose finished
8:15 a.m. Ingestion of 100 cc. water
8:30 a.m. Ingestion of 100 cc. water
8:45 a.m. Ingestion of 100 cc. water
9:00 a.m. Emptied bladder. Urine volume for two hours. Ingestion of 100 cc. water

9:07 a.m. Intravenous injection begun of 10.08 grams of sucrose (0.13 gm. kgm.) in 20.15 per cent solution, 50 cc.
9:27 a.m. Injection finished
9:34 a.m. Ingestion of 200 cc. water
9:47 a.m. Emptied bladder. Urine volume 47 minutes
9:49 a.m. Blood withdrawn from vein of arm
10:00 a.m. Ingestion of 200 cc. water
10:17 a.m. Emptied bladder. Urine volume 30 minutes, first collection period
10:19 a.m. Blood withdrawn from arm vein

TABLE 1

Comparison of simultaneous clearances of sucrose, xylose, urea and inorganic sulphates

SUBJECTS	AGE, YEARS	WEIGHT, KGM.	HEIGHT, CM.	BODY SURFACE, SQUARE METERS	EXPERIMENTAL PERIOD, MINUTES	URINE						BLOOD PLASMA AND SERUM†				PLASMA AND SERUM CLEARANCES			
						Minute volume (V) cc.	Specific gravity*	Sucrose (U), gm. per cent	Xylose (U), gm. per cent	Urea (U), gm. per cent	Sulphate (U), gm. per cent	Sucrose (P), mgm. per cent	Xylose (P), mgm. per cent	Urea (P), mgm. per cent	Sulphate (S), mgm. per cent	$\frac{U}{P} \times V$ Sucrose,	$\frac{U}{P} \times V$ Xylose,	$\frac{U}{P} \times V$ Urea,	$\frac{U}{S} \times V$ Sulphate,
C	32	81.4	177.5	1.97	40	3.35	1.022	1.770	1.823	0.647	0.031	53.0	62.0	26.0	2.45	112	98	83	42
					38	8.5	1.008	0.370	0.750	0.214	0.012	32.5	64.3	29.0	2.55	97	99	63	40
K	48	77.0	173.7	1.90	30	2.3	1.022	1.930	2.010	0.595	0.047	39.5	46.6	17.5	2.2	112	99	78	49
					30	5.9	1.006	0.500	0.594	0.223	0.016	27.0	39.1	21.0	2.1	109	90	63	45
M	30	70.0	173.7	1.84	30	3.4	1.016	1.540	1.565	0.310	0.016	48.5	56.5	16.5	2.5	108	94	64	22
					30	2.17	1.015	1.540	2.620	0.473	0.025	34.0	60.3	15.5	2.4	98	94	66	23
P	39	74.5	172.5	1.88	30	6.4	1.008	0.720	0.459	0.278	0.017	57.0	39.9	28.0	3.5	81	73	64	31
					30	8.9	1.004	0.370	0.300	0.180	0.012	41.0	40.8	26.0	3.5	80	65	62	30
B	36	78.2	176.2	1.95	30	7.73	1.007	0.665	0.943	0.184	0.0127	49.5	78.7	16.0	2.5	104	93	89	39
					30	10.7	1.004	0.309	0.594	0.126	0.0082	33.5	70.7	15.0	2.6	99	90	90	34
Mean						5.9										100	89.5	72.2	35.5

* Specific gravity determined by means of the pyknometer.

† Mean value obtained at beginning and end of experimental period. Albumin (qualitative test) always negative. All male subjects. Subjects K, P and B no diarrhea. Subjects C and M moderate diarrhea.

10:34 a.m. Ingestion of 200 cc. water
10:47 a.m. Emptied bladder. Urine volume 30 minutes, second collection period
10:49 a.m. Blood withdrawn from arm vein

Chemical methods. Venous blood was prevented from coagulating by means of purified heparin, and the plasma was separated in the centrifuge immediately. For the determination of sucrose and xylose, tungstic acid filtrates were used, while in the case of urine, appropriate dilutions served. The plasma filtrates were prepared according to Wu; that is, 1 volume of plasma (4 cc.) was delivered into eight volumes of water and precipitated by the addition of a half volume each of $\frac{2}{3}$ normal sulphuric acid and 10

per cent sodium tungstate. The pH of the filtrates was about 4.5. The reducing power of the filtrates and dilutions of urine was determined with a modified Shaffer-Hartmann reagent of composition closely similar to "Reagent 50," recently described by Shaffer and Somogyi. The iodometric titrations were done with 0.01 normal sodium thiosulphate, delivered from a 10 cc. microburette equipped with a Shohl needle tip. Duplicate analyses (boiled separately) checked within usually 0.01 cc. or at most 0.02 cc. The titrations of the unknowns were subtracted from blanks run simultaneously. These also usually agreed exactly or at most within 0.02 cc. The error in a single determination may be, therefore, as large as 0.04 cc. of 0.01 normal thiosulphate, which is equivalent to about 2 mgm. of glucose. The relationship between glucose and thiosulphate was determined with Bureau of Standards glucose, and a curve constructed from which the blood sugar values were read. This curve was checked at frequent intervals.

Sucrose. The reduction of plasma filtrates and dilutions of urine before inversion was subtracted from that after inversion, as read from the glucose curve, and multiplied by a factor experimentally established for the conditions of the analysis, 0.960. The inversion was accomplished as follows: Ten cubic centimeters of filtrate or 6 cc. of dilution of urine containing not more than 0.5 per cent sucrose were placed in a tube marked at 12 cc. and brought to a volume of 10 cc. The pH was adjusted to 4.0 to 4.6 with bromcresol green, the tube was brought to 40°C., and 0.5 cc. of a 1 to 10 dilution of a stock solution of invertase was added, after which the incubation at 40°C. was continued for thirty minutes longer (7). A blank on the invertase was conducted similarly. The invertase was prepared from pressed yeast, according to the alcohol method of Euler and Svanberg, a procedure which yielded active preparations of low reducing power with a minimum of time and labor. After incubation the contents of the tubes were diluted to the 12 cc. mark and, in the case of the urine, further diluted for the determinations of sugar. (Dilution first to the plasma filtrate concentrations, and then inversion, yielded the same results in urine.) In control experiments solutions of sucrose as strong as 1 to 2 per cent were completely inverted under the conditions described. The reduction of the invertase itself was small and could be neglected in the analyses of urine. As the copper reagent contained ample buffer capacity, no further adjustment of pH after inversion was necessary.

Xylose. The plasma filtrates and corresponding portions of urine, diluted to approximately the same concentration of xylose, were incubated with washed yeast fifteen minutes at 35°C., in the proportion of 10 to 12 cc. of filtrate, or dilution, to the packed yeast obtained by centrifuging down a corresponding volume of 10 per cent suspension of washed Fleischmann's yeast. This is a modification of Somogyi's technic as devised by Spannuth

and Power, and was found to remove glucose and sucrose quantitatively, while leaving an average of 92 per cent of the xylose present. The recovery of xylose was the same whether freshly washed yeast was used, or whether suspensions were used which had been preserved one to seven days in the refrigerator, the water being poured off and replaced each day with fresh water. As the reducing power of xylose with the reagent used is slightly less than that of glucose, a special curve from which the xylose sugar values were read was constructed. The copper reagent is particularly sensitive to the nonfermentable reducing substances in plasma; accordingly appropriate correction for these substances was made. This correction was found to be remarkably constant for the different subjects and to be unaffected by the ingestion of oatmeal. Correction for nonfermentable reducing substances in urine was not made, as the urines were considerably more dilute in this respect than usual. Neglect of this factor would tend to make the xylose clearances, as calculated, somewhat too high.

Accuracy of determinations of sugars. The recovery of known amounts of sucrose and xylose when added in solution to pooled oxalated plasma in place of some of the water required for the preparations of the 1 to 10 tungstic acid filtrates, has been accurate, usually to 1 to 2 mgm. The variability in experiments of this sort is illustrated by the data in table 2, which show that in eight recoveries of added sucrose at the 25 mgm. level, two were in error more than 1 mgm. or more than 5 per cent, and at the 50 mgm. level, one was in error more than 2 mgm. or more than 5 per cent, whereas in twelve recoveries of 50 mgm. of xylose, one deviated 2.5 mgm., or 5 per cent. These instances represent the extremes, while the mean differences from the quantities of sugars added were, of course, much smaller. A similar but somewhat better order of accuracy was experienced in the recovery of these sugars from urine or from pure sugar mixtures.

Urea. The urease aeration and titration method of Marshall as modified by Van Slyke and Cullen was used. Samples of plasma of 3 cc. and urine equivalent to 0.5 to 1.0 cc. were incubated with the enzyme. The ammonia correction for urine was estimated by aerating samples of 5 cc.

Inorganic sulphate. Inorganic sulphate in serum was determined by a modified benzidine method, the details of which have not been published. In brief, this consisted of precipitating the sulphate as the benzidine salt from trichloracetic acid filtrates essentially according to Hubbard. The resulting precipitate was then titrated hot with 0.001 normal sodium hydroxide, with phenol red as indicator. With appropriate precautions the use of such weak alkali is entirely feasible, and we believe the results to be somewhat more trustworthy than the dichromate oxidation method previously proposed by Power and Wakefield. In the more dilute samples of urine encountered, the sulphate was precipitated and titrated as in the determinations of serum; in the more concentrated samples 0.005 normal

sodium hydroxide was used for the titration. The recovery of sulphate from urine by the technic utilized has compared favorably with the gravimetric method.

Estimation of clearances. In determining the clearance of a given substance in terms of cubic centimeters of plasma or serum cleared per minute, the simple formula $\frac{U}{P \text{ or } S} \times V$ was used. U = concentration in urine; P or S, concentration in plasma or serum; V, minute volume of urine. The plasma or serum value was the mean of the concentrations obtained at the beginning and end of the experimental period.

RESULTS. In comparing the excretion of sucrose to xylose it will be noticed that the mean of the sucrose clearances is approximately 10 per cent greater than that of xylose (table 1). The variation for ten sucrose clearances is 80 to 112, with a mean of 100; for xylose 65 to 99 and a mean of 89.5. There is only a single xylose clearance which is greater than that of sucrose, that occurring in the second period in subject C (table 1). The xylose clearance was 99 and the sucrose clearance, 97. The mean of the differences between the determinations of sucrose and of xylose was found to be 10.5 ± 1.2. The difference is thus more than eight times its probable error, and therefore, of definite significance statistically. In subject P (table 1) both the sucrose and xylose clearances are lower than in the other four subjects. This individual we think had normal kidneys, but has never excreted, in a long series of experiments with sucrose, as much of this sugar as other normal persons. He also showed a similar low xylose clearance in another experiment when xylose alone was taken. The fact that the plasma concentrations of xylose formed a plateau, while those for sucrose were gradually falling during the period of clearance might lead one to surmise that the difference in plasma levels was the reason for the variation in their respective clearances. This is unlikely, for in two unpublished experiments dealing with subjects K and P, in which the level of the plasma sucrose was held at a plateau, the sucrose clearances were the same as in the present series. Our difference between sucrose and xylose clearances is distinctly less than that obtained by White and Monaghan in dogs, but slightly greater, although comparable, to those of Smith and coworkers in dogs and in man after administration of phlorizin (1).

The variations of the urea clearance from 62 to 90, with a mean of 72.2 are comparable with previous results. The relatively low clearance for inorganic sulphate, 22 to 49, mean 35.5, is similar to that obtained by Hayman and Johnston and by Macy. Cope also has reported sulphate clearances relatively lower than urea. However, it should be pointed out that these sulphate clearances were estimated under strictly controlled conditions, in the morning hours. Macy has shown that an individual on his usual diet excretes considerably more inorganic sulphate in the

early evening than during the rest of the day. Also, experiments have been reported, both on man (4) and on animals (12), (19), in which a considerable amount of sulphate was injected intravenously; the clearances were relatively much higher than in the present series and approached that of urea and sometimes even that of creatinine. It is also of interest

TABLE 2

*Recovery of sucrose and xylose after addition to plasma**

Quantities in terms of milligrams in 100 cc. of plasma

EXPERIMENT	SUCROSE ADDED		XYLOSE ADDED
	25.0	50.0	50.0
	SUCROSE RECOVERED		XYLOSE RECOVERED
1	24.5	51.8	49.6
	24.0	49.0	50.2
			49.6
2	25.0	51.8	50.7
	26.4	49.9	50.7
			50.2
3	25.4	52.8	50.7
	23.3	51.8	47.5
			48.6
4	24.0	48.5	50.7
	25.4	49.9	50.7
			50.7
Mean recovery	24.64	50.7	49.99
Mean difference from amount added	−0.36	+0.7	−0.01
Standard deviation	0.97	1.61	0.98
Extremes	−1.7 to +1.4	−1.5 to +2.8	−2.5 to +0.7

* The figures show recovery of sucrose after addition alone, and with 50 mgm. xylose, respectively; of xylose after addition alone; with 25 mgm. sucrose, and with 50 mgm. sucrose, respectively.

that the addition of sucrose and xylose to the normal renal excretory substances does not appear to alter the clearances of urea and of sulphate.

The largest variation of the clearances in the same individual is approximately 10 per cent for sucrose, xylose and sulphate, but much greater, or 25 per cent, for urea. This greater fluctuation of the urea clearance, when compared to that of sucrose, and xylose, has been pointed out by

Jolliffe, Shannon and Smith in dogs; that of urea when compared to sulphate in man, by Macy.

COMMENT. In this comparatively small series of experiments, an attempt has been made to determine the relative renal excretion of four substances under carefully controlled conditions. Care also has been taken in selecting the chemical methods and in estimating the limits of their accuracy.

The range of normal renal excretion must be considerable. It is known that the number of units in active function fluctuates from time to time, and that variations in atmospheric temperature and diet (2), (8), (9), (15), and in the quantity of ingested water, also alter renal function. In devising the present experiments we endeavored to control as many of these factors as possible. A good flow of urine was always obtained. Digestive factors were limited to the ingestion and absorption of water and xylose, also to the digestion of oatmeal and absorption of its digestion products. Atmospheric temperature varied little, as the experiments were carried out in the summer. Even under these conditions, our results indicate that physiologic processes may vary quantitatively within a comparatively short time.

Some earlier results, reported by us last July (10), the findings of Jolliffe and Chasis, and Smith and his co-workers, and our present results, show that in normal men the relative clearances of creatinine, the sugars, sucrose and xylose, urea and inorganic sulphate are approximately 150, 100, 70 and 35.

The urea clearance is distinctly more variable than that of the sugars and sulphate. One reason at least is that the latter substances are less influenced than is urea by variations in excretion of water. The clearances of sucrose and xylose are closely comparable, but in our results that of sucrose is slightly greater than that of xylose. As we have pointed out, this difference is definitely greater than its probable error. This finding, however, does not rule out the possibility of unrecognized systematic errors, which might explain the small differences in the experimental results. Therefore, we believe that the clearance of sucrose or xylose has the same physiologic significance.

SUMMARY

Among normal men the clearance of sucrose was found to be slightly greater than that of xylose. The clearance of urea is definitely less than that of the sugars, and the clearance of inorganic sulphate less than that of urea.

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METABOLIC AND IRRITABILITY CHANGES IN NUTRITIONAL MYOPATHY OF RABBITS AND DUCKS

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Although many investigations have been concerned with the metabolism of normal muscle, very little is known about the physiological changes in diseased muscles. Hitherto, because of the rarity of muscular disorders in animals and man, studies of diseased muscles have been even less common. The nutritional muscular dystrophy in guinea pigs, rabbits, and ducks described by Goettsch and Pappenheimer (4, 5) offers an opportunity for determining the chemical and physiological changes which accompany the various stages of primary muscle degeneration. The fall in creatine content in the diseased muscles of rabbits has recently been described by Goettsch and Brown (6).

The present communication is concerned with the oxygen consumption, and with the irritability of normal and diseased muscles of ducks and rabbits. The pathological changes in the two species are very similar. However, it has not been established that the same nutritional factors are responsible.

METHOD. *A. Oxygen consumption.* The oxygen consumption was measured in Fenn volumeters with a slight modification for mammalian tissue experiments as suggested by Professor Fenn. The graduated capillary is placed below the stopcocks. The stopcocks rest in a vertical instead of a horizontal position, so that they lie perpendicular to the axis of rotation. Then the centrifugal force due to shaking has a minimum displacing effect upon the stopcocks. The muscles were cut with fine scissors parallel to the fiber bundles into thin strips and placed in Ringer's solution. This consisted of 0.9 per cent NaCl, 0.023 per cent KCl, 0.022 per cent $CaCl_2$, and a phosphate buffer of pH 7.44 (1) containing 10 mgm. of P per 100 cc. After sectioning the muscle was rapidly weighed on a Roller-Smith torsion balance and placed into the experimental bottle containing 1 cc. of the Ringer-phosphate solution. An inset containing a few drops of 0.2 N NaOH absorbed the CO_2 produced. Pure oxygen was flushed through the bottle for about five minutes through a stopcock attached to it. Each bottle had a capacity of about 15 cc. The temperature was 37.5° ±0.005°C. The tissues weighed between 50 and 150 mgm.

The respirometers were shaken through an excursion of 6 cmm. 150 times a minute. The oxygen consumption was followed for periods of two to eight hours. At the end of the experiment the tissue was fixed in Zenker's solution and sectioned for microscopic study.

B. Irritability. The "strength-duration curves" of the muscles were determined by the condenser discharge method of Lapicque. The resistances were arranged with 17,000 ohms in series with the tissue. The latter was shunted by a 3,000 ohm resistance so that variation of the tissue resistance between 2,000 and 50,000 ohms varied the total resistance by not more than 15 per cent from 20,000 ohms. The electrodes were platinum, constantly 1 cm. apart. A microammeter placed in series with the condensers and the electrodes could always be observed as the muscle was stimulated.

EXPERIMENTAL. The muscles were taken from rabbits that had been on stock diets, or on the experimental diets 11, 13, 32, and 33,[1] and from ducks on stock diet 20 or experimental diet 108.[1] The adductor muscles of the thigh were usually chosen for study. The other muscles used are noted in the tables. The animals were anesthetized with ether, amytal, or local anesthesia and the desired muscle exposed. No differences in the effects from these anesthetics were noted. The electrodes were placed directly into the muscle and the threshold potential for a 1.1 m.f.d. condenser was determined. The potential was then progressively increased and the minimum capacity for stimulation was determined. Chronaxie was determined directly. Usually the right and left adductor were studied in the same animal. Then the muscle was excised and a part was analyzed for creatine. The remainder was sectioned, weighed, and placed in the respirometer. About 15 minutes elapsed between the irritability and the oxygen consumption measurements.

RESULTS. Since we were unable to find any data in the literature upon the oxygen consumption of normal rabbit and guinea-pig muscle, the metabolism of a series of dog and rat skeletal muscles was studied to compare the results obtained by our technique with those reported on similar muscles in the literature. Three determinations for dog rectus abdominis gave values of 3.1, 4.6, and 4.6 cmm. O_2/gm./min. Four observations on rat diaphragm gave values of 15.8, 17.0, 14.5, and 15.1 cmm. O_2/gm./min. These observations agree with those of other authors (7, 8, 9, 10, 12).

The oxygen consumption of normal rabbit muscle is found to be considerably lower than that of dog or rat as seen in table 1. That the rate of oxygen consumption of the muscles was fairly constant during an experiment can be seen in figure 1 where the metabolic rates of normal and dystrophic rabbit skeletal muscle are compared.

[1] For composition of these diets 11, 13, 108, and 20, see (4 and 5).
Diets 32 and 33 are modifications of diet 11 and produced identical muscle changes.

The dystrophic rabbit skeletal muscle is found to have a much higher rate of oxygen consumption than the normal (table 2, fig. 1). This may be as much as 200 to 400 per cent higher than the normal metabolic rate. A comparison of the metabolic rates shows considerably more variation among the diseased muscles than the normal. The reason for this is obvious. There is a great deal of variation in the intensity and character of the pathological changes in the diseased muscles. However, there is a strong

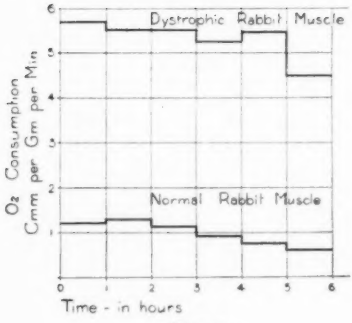

Fig. 1

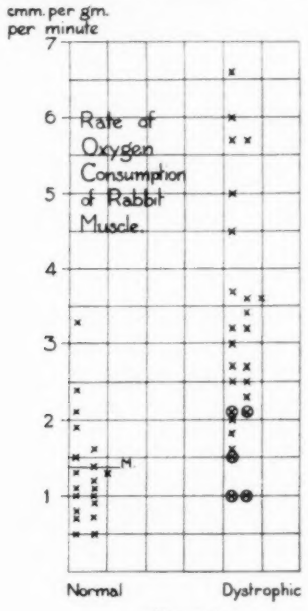

Chart 1

Fig. 1. The different rates of oxygen consumption of normal and dystrophic rabbit muscle. The rates are uniform for a period of several hours.

Chart 1. The different rates of oxygen consumption of a series of normal and of dystrophic rabbit muscle. *M* signifies the mean of the normal muscles; it is 1.4 cmm./gm./min. (x)—These muscles had normal creatine values (see table 3), but are included in the dystrophic series because they show early histological changes.

suggestion of an inverse relationship between the creatine values and the rates of oxygen consumption in the diseased muscles. This is especially striking in rabbit 75 in which biopsies taken at successive intervals show metabolic changes that are exactly opposite to the changes in creatine content. The rates of oxygen consumption and the creatine contents of muscle removed on January 1, 1933, were 2.1 and 2.5 cmm./gm./min. and 486 mgm./100 grams; on March 8, 1933, they were 5.0 cmm./gm./min.

and 138 mgm./100 grams; and on March 18, 1933, 3.4 cmm./gm./min. and 391 mgm./100 grams respectively.

The "strength-duration" relationships for electrical excitation of these muscles are also altered. On the whole there is a much greater change in the rheobase than in the chronaxie. The mean rheobase for the normal

TABLE 1

Rheobase, chronaxie, and creatine content of normal rabbit muscle

Irritability determined in situ; oxygen consumption measured after excision of these muscles

RABBIT NUMBER	AGE	DIET	MUSCLE	OXYGEN CONSUMPTION	CREATINE	IRRITABILITY	
						Rheobase volts	Chronaxie 10^{-2} seconds
				cmm./gm./min.	*mgm./100 gm.*		
64	Adult	Stock	Triceps	2.1			
				1.3	470		
				0.7	439		
				1.0	446		
	Adult	Stock	Rectus femoris	0.8			
				1.1			
				1.0			
	Adult	Stock		1.2			
				1.1			
				0.9			
				3.3			
148*	4 mos.	33	Adductor	1.4	454	14	0.60
K32B	1 mo.	Stock	Adductor	1.5	357	3.5	0.60
K32C	1 mo.	Stock	Adductor	0.7	308	10	0.32
K12G	8 mos.	Stock	Adductor	0.5	484	16	1.40
K62G	8 mos.	Stock	Adductor	1.3	585	5	0.70
				1.6	497	6	0.70
K62F	8 mos.	Stock	Adductor	1.9	495	4	0.70
				2.4	510	4	1.00
140	4 mos.	Stock	Adductor	0.5	547	18	0.78

* Rabbit 148 on diet 33 included in table because it showed neither histological nor chemical changes in its muscles.

rabbit muscle was 8.9 volts and the chronaxie 0.75 sigma; while the mean rheobase and chronaxie of the diseased muscles were 21.4 volts and 1.15 sigma respectively.

Included in the table of diseased rabbit muscles are rabbits 137 and 149. Although their metabolic rates and creatine values are in the normal range their muscles showed slight alterations histologically. The rheobase and chronaxie of the former were above the normal values, but those of the latter were normal. (See chart 1.)

In table 3 are listed the rate of oxygen consumption, the creatine con-

TABLE 2

Chronaxie, rheobase, creatine content, and histology of dystrophic rabbit muscle

TABLE 2

Chronaxie, rheobase, creatine content, and histology of dystrophic rabbit muscle

Irritability measured in situ; oxygen consumption after excision

RABBIT NUMBER	AGE	DIET	DAYS ON DIET	MUSCLE	OXYGEN CONSUMPTION	CREATINE	HISTOLOGY			IRRITABILITY	
							Necrosis	Cellular infiltration	Fibrosis or fat replacement	Rheobase volts	Chronaxie 10^{-3} seconds
					cmm./gm./min.	*mgm./100 gm.*					
26	Adult	13	141	Quadriceps	2.1	527	+	+	−		
					3.2	390	++	++	−		
33	Adult	13 and 50 gm. lettuce	254	Extensors of leg	5.7	215	+	+	++++		
					6.0	102	++	+	++++		
					3.7	150	++	++	+++		
31	Adult	13 and 50 gm. lettuce	261	Pectoral	4.5	177	++	+++	++		
30	Adult	13 and 50 gm. lettuce	266	?	5.7						
					3.6						
				Gluteus		286	+	+	+		
				Gastrocnemius		119	++	+	+++		
				Triceps		77	+	++	++++		
25	Adult	13	297	?	6.6						
				Gluteus		200	++	+	+++		
				Gastrocnemius		173	++	+	++		
				Triceps		162	++	+	+++		
78	Young	11 and 10% gelatin	166	Triceps	3.6		+++	+	+++		
				Mixed muscles		119	++	+	++++		
75*	Young	11	331	Adductor	2.1	486	±	+	−		
					2.5						
				Adductor	5.0	138	++	+	++++		
				Adductor	3.4	391	++	−	++++		
116	Young	11	230	L. Adductor	2.7	345	++	+	++		
154	2 mos.	11	23	Adductor	2.0	326	++	++	0	18	0.36
129	3 mos.	11	41	Adductor	1.8	297	+++	++	+	26	2.20
133	3 mos.	32	42	Adductor	1.5	475	++	+	−	5	1.08
					1.6	258	+	+	−	30	0.40
132	3 mos.	11 and alfalfa	43	Adductor	3.2	177	+++	+++	+	36	0.36
					2.3	217	+++	+++	+	42	1.80
143	3 mos.	11 and alfalfa		Adductor	2.7	336	+	−	−	8	2.80
152	3 mos.	11 and alfalfa		Adductor	3.0	380	++	+	−	26	0.76
145	4 mos.	33		Adductor	2.5	257	++	+	0	20	0.50
137	4 mos.	32		Adductor	1.0	408	+	±	0	15	2.00
149	4 mos.	33		Adductor	1.0	431	±	0	0	9	0.38

* Rabbit 75 had three biopsies taken on January 1, March 8, and March 18, 1933. See text for findings on these days. There is a striking inverse relationship between the rate of oxygen consumption and the creatine content of the muscle.

tent, rheobase, and chronaxie of normal duck muscle. The metabolic rate is more like that reported by the authors mentioned for dog muscle. It is about three times as great as that of normal rabbit muscle. The rheobase and chronaxie values are not unlike those found for rabbit muscle (table 1). The creatine content is also similar to that of rabbit skeletal muscle.

Contrary to the findings in rabbits, the respiratory rate of dystrophic duck skeletal muscle is no different from that of normal duck skeletal muscle. There are distinct alterations in the strength-duration relationships, however. The changes in chronaxie and rheobase parallel each other. Included in table 4, in which the dystrophic duck muscles are recorded, is duck 1929 which shows neither irritability nor histological changes. However, the

TABLE 3

Rheobase, chronaxie, and creatine content of normal adductor duck muscle

Ducks 16 to 22 days old on stock diet 20. Irritability and oxygen consumption measured as in previous experiments.

DUCK NUMBER	DAYS ON DIET	OXYGEN CONSUMPTION	CREATINE	IRRITABILITY	
				Rheobase volts	Chronaxie 10^{-3} seconds
		cmm./gm./min.	*mgm./100 gm.*		
1933	16	3.0	547	11	0.2
		3.0	517	3	0.26
1935	19	5.7	374	9	2.0
		4.7	350	26	0.94
1938	19	3.4	373	11	0.5
1936	20	6.2	494	14	0.24
1937	20	2.6	466	6	0.66
		2.9	550	18	0.74
1932	22	4.8		11	0.68
		5.9	424	13	0.26

creatine content was diminished even though not to the same degree as was observed in most of the other muscles. Duck 1919, on the other hand, showed widespread necrosis of its muscle fibers histologically, yet its creatine content,[2] rheobase, and chronaxie were within the normal range.

DISCUSSION. A statistical analysis of the metabolic and irritability changes is futile because of the great variation in degree and type of change in the muscles. Nevertheless, it is obvious that the metabolic rate in the diseased muscle is greatly increased. An explanation of this increased rate of oxygen consumption observed in dystrophic rabbit muscle cannot be attempted at this stage of the problem. That increased oxygen consumption may be associated with loss in irritability is known in the case of the

[2] A larger sample of muscle, however, gave a creatine value of 243 mgm. per 100 grams of wet muscle.

reversible change produced in frog skeletal muscle by sugar solutions (3). The fact that the chronaxie is slightly increased may be due to a partial loss of irritability in some of the muscle fibers, while others have completely lost their ability to respond to an electrical stimulus. The increased rheobase is changed to a far greater extent (135 per cent) than the chronaxie (48 per cent). Certain exceptions to this are seen in table 2 (rabbits 129, 143, and 145).

TABLE 4

Oxygen consumption, rheobase, chronaxie, creatine content, and histology of dystrophic duck muscles

Ducks 15 to 20 days old on diet 108. Determinations as in previous experiments

DUCK NUMBER	DAYS ON DIET	MUSCLE	OXYGEN CONSUMPTION	CREATINE	HISTOLOGY			IRRITABILITY	
					Necrosis	Cellular infiltration	Fibrosis fat replacement	Rheobase volts	Chronaxie 10^{-3} seconds
			cmm./gm./min.	*mgm./100 gm.*					
1921	15	Adductor	5.4	90	++++	+++	0	23	2.0
			4.3	165				56	2.0
1928	16	Adductor	1.9	118	++++	++	0	70	
			5.9	113				29	0.7
1926	17	Quadricep	5.5	139	++++	+	0	9	5.0
			4.8	193				27	0.32
1919	18	Adductor	3.1	386	++++	0	0	15	0.92
			4.2					7	0.58
1922	19	Adductor	5.6	276	++++	++		22	1.8
			5.1	169					1.8
1927	19	Adductor	3.9	215	++++	+	0	29	1.2
1929	20	Adductor	3.1	298	−	−	−	18	0.68
			3.8	301				6	0.68

Since the muscles were stimulated directly the changes observed cannot be due to alterations of nerve conduction. Furthermore the nerve endings have been shown to be unaffected in this disease (11).

The increased metabolic rate associated with the decreased creatine content in the diseased rabbit skeletal muscle is paralleled by the normal relationship between normal resting cardiac and skeletal muscle of the frog. The oxygen consumption of the former is much higher than that of the latter (13), while the creatine content is much lower (2).

The rôle played by the infiltrating cells in the altered respiratory rate of the tissue has not been determined. However, an analysis of the tables shows that no correlation exists between the degree of infiltration and the

rate of oxygen consumption. The respiration of leucocytes is known to be very much greater than that of muscle.

At present it is impossible to explain the physiological differences observed between dystrophic duck and rabbit skeletal muscle. The diets used in these experiments for producing the diseases were quite different, and even though the pathological alterations were similar, nutritional factors concerned were probably not identical. The loss of irritability in the two species seems to be different. In the case of the duck muscles both chronaxie and rheobase seem to change equally, suggesting a more graded loss of irritability for the individual fiber, than in the rabbit muscle.

SUMMARY AND CONCLUSIONS

In nutritional muscular dystrophy of the rabbit the rate of oxygen consumption of the diseased muscle may be increased as much as 400 per cent above the mean of the normal rate. The rheobase and chronaxie of the dystrophic muscles is greater than normal. The rheobase is increased much more than the chronaxie.

On the other hand, in the nutritional myopathy of ducks the rate of oxygen consumption of the diseased muscles is no different from that of normal ducks. The rheobase and chronaxie of the dystrophic muscles is greater than normal. The degree of change in chronaxie and rheobase is the same.

I am greatly indebted to Dr. Marianne Goettsch for the privilege of including the data on the creatine determinations in these experiments and to Prof. A. M. Pappenheimer for his interest and suggestions throughout this study.

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THE EXTRACTION OF THE ADRENAL CORTICAL HORMONE FROM THE INTERRENAL BODY OF FISHES[1]

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It is generally recognized, on the basis of anatomical and embryological studies, that the segmentally arranged and paired chromaffin bodies of certain elasmobranch fishes (shark, dogfish, ray, etc.) are homologous with the medulla, while the interrenal body is homologous with the cortex of the adrenal body as it is constituted in other animal species (Dean, 1923).

Direct evidence exists for the physiological identity in function of the chromaphil bodies and the adrenal medulla. Thus Vincent (1897, 1898a) showed that the saline extracts of these bodies in the dogfish had vasoconstrictor and pressor activity similar to that of extracts of the adrenal medulla. Similar extracts prepared from the interrenal bodies manifested no such actions. Vincent's observations were confirmed by Biedl and Wiesel (1902). Lutz and Wyman (1927) have also demonstrated the mydriatic action of extracts of the chromaphil tissue of certain elasmobranchs, which is indicative of the elaboration of epinephrine by these bodies. Extracts of the interrenal body, on the other hand, were free of any sympathicomimetic substance.

Our knowledge of the physiological function of the interrenal body, on the other hand, is based solely on extirpation experiments. Vincent (1898b) concluded that the interrenal body is not essential to life in the eel, a fact that was shown by Giacomini (1908) to be attributable to the presence of additional interrenal tissue in the anterior edge of the pronephros and attached to the cardinal veins. Biedl (1913) demonstrated that removal of the interrenal bodies in the skate and the torpedo results in symptoms (weakness, pallor, cachexia, etc.) and ultimate death analogous to that observed after removal of the adrenal cortex in higher animals. Kisch (1931) has confirmed and extended these observations.

The operative difficulties involved in performing interrenalectomy in fishes renders conclusions based on extirpation experiments of doubtful value. Less equivocal evidence is obtainable by actually extracting the

[1] Aided by grants from the National Research Council and the Hartley Corporation, for which we wish to express our appreciation.

cortical hormone from the interrenal body. Cleghorn (1932), however, failed to find this hormone in extracts of the interrenal body but his negative findings may be attributed, as he has indicated, to the rapidity with which the hormone is destroyed by autolytic processes, to the unsatisfactory nature of the method used for its extraction, and to the lack of an assay method capable of determining the presence of small amounts of the hormone.

The success of our own efforts may be attributed to the avoidance of these three sources of difficulty.

EXPERIMENTAL. Extracts were prepared from the interrenal bodies of skates. These fish were caught on trawls in the St. Croix River (at St. Andrews, N. B.) and in Passamaquoddy Bay. They comprised the three common species indigenous to these waters (Bigelow and Welsh), namely, the Barn-door (*Raja Stabuliforis*, Garman), the Big Skate (*Raja diaphanes*, Mitchell), and the Little Skate (*Raja erinacea*, Mitchell). In order to avoid the enzymatic destruction of the hormone which probably occurs rapidly and at relatively low temperatures in fish tissue, the interrenals were dissected from the living fish, after transection of the spinal cord. The glands were immediately dropped into about twenty times their volume of acetone and reduced to tiny particles by cutting with scissors under acetone. The combined acetone-gland mixture was diluted with an equal volume of distilled water and an extract was prepared according to the procedure described (Grollman and Firor, 1933b) for adrenal glands. In view of the absence of epinephrine in interrenal tissue, the stage of the process involving the removal of this substance was omitted.

The extract thus obtained was tested by the rat-assay method previously described (Grollman and Firor, 1933a, b; 1934). A typical experiment is reproduced in figure 1. The upper continuous curve shows the effect of a daily dose of 0.2 cc. of the extract derived from approximately 2 grams of tissue.[2] This dose permitted growth and prolonged the survival of doubly adrenalectomized rats in a way similar to that observed with extracts of the adrenal cortex. The lower interrupted curve shows the failure and early death of control animals receiving an equivalent dose of 0.9 per cent saline. Other experiments gave essentially the same results as those shown in figure 1.

DISCUSSION. The above described results afford direct evidence for the homologous physiological function of the interrenal body and the adrenal cortex. The fact that the homologues of the medulla and the cortex occur as discrete and separate bodies in the elasmobranch fishes and the fact that no epinephrine-like substance has been found in the

[2] It was impractical to weigh accurately the fresh interrenal tissue and hence the gland equivalent of the extract as given above is a rough approximation based on the weight of the dry gland remaining after extraction of the hormone.

interrenal would speak against the view that epinephrine as claimed by Kendall (1933) is one of the molecular constituents of the adrenal cortical hormone. Were this the case, one certainly would expect extracts of the interrenal to manifest sympathicomimetic activity. To explain teleologically the significance of the close apposition of medulla to cortex in higher species we suggest that epinephrine, by virtue of the relatively high oxidation-reduction potential which it manifests and its highly unstable oxidant (Ball and Chen, 1933), is stabilized by the highly reducing constituents of the adrenal cortex, particularly by ascorbic acid (Szent-Györgyi, 1933).

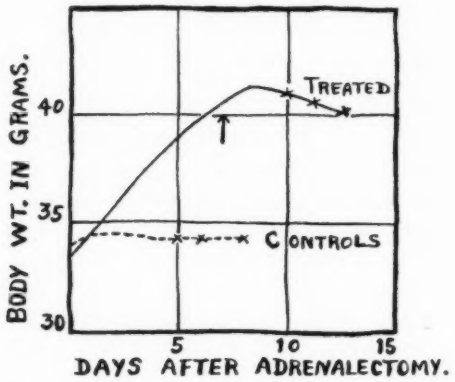

Fig. 1. The growth-curve of doubly adrenalectomized rats. The upper continuous curve shows the growth of rats receiving the extract of the interrenal. Treatment ceased on the seventh day at the point indicated by the arrow. The lower curve shows the absence of growth in the control animals. The crosses (x) indicate the death of an experimental animal.

We are indebted to Acting-Director Laim and his staff for their courtesy in permitting us to collect some of the necessary materials at the Atlantic Biological Station, St. Andrews, N. B. We are especially indebted to Mr. M. F. H. Freedman of McGill University who kindly collected some of the material for us.

SUMMARY

An extract of the interrenal body of the skate was prepared which maintained normal growth in bilaterally adrenalectomized young rats. This proves physiological homology of the interrenal and the adrenal cortex. The significance of the morphological relation of adrenal cortex and medulla is discussed.

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THE INTRINSIC REGULATION OF THE BLOOD VESSELS OF THE MEDULLA OBLONGATA

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This work is a continuation of previous efforts (Schmidt, 1928) to identify the factors involved in the intrinsic regulation of cerebral vessels and to evaluate their importance in the regulation of the activity of vegetative functions, particularly respiration. As a result of the earlier experiments (1928) we concluded that the cerebral vessels are subject to an intrinsic chemical regulation of such a nature that the influence of alterations in chemical composition of the blood would be antagonized by alterations in blood-flow through the respiratory center. This conclusion was based upon Gesell's (1923) conception of respiratory control as a function of the internal chemical environment of the cells of the respiratory center, according to which the metabolic activity and blood-supply of the cells of the center are prominent factors in determining the activity of the center. Subsequent work by Heymans and Bouckaert (1930) and Bouckaert and Heymans (1933) has led them to conclude that the respiratory center is unaffected by physiological changes in its blood-supply, and that cerebral blood-vessels have no capacity for intrinsic regulation of their tone; according to them, the phenomena which had previously been attributed to alterations in blood-supply of the respiratory center are actually due entirely to reflexes from the carotid sinuses. Reinvestigation of the question from the standpoint of carotid sinus reflexes enabled us to confirm many of Heymans' observations (Schmidt, 1932a). At the same time, however, we were able to demonstrate respiratory responses of the sort demanded by Gesell's theory under conditions such that known reflexes were excluded, and our belief in the existence and physiological importance of an intrinsic regulation of blood-vessels supplying the vegetative centers was reaffirmed (Schmidt, 1932b). It seemed probable to us that differences in tone of these blood-vessels were responsible for the different results and opposite conclusions attained by Heymans and ourselves. Additional information on this subject was therefore highly desirable. The medulla was chosen for first investigation, not only because important vegetative centers are concentrated here, but also because its functional activity is demonstrably retained under nar-

cosis or decerebration; our earlier experiments (1928) had given us reason to suspect that the intrinsic regulating capacity of cerebral vessels disappears when activity of central nerve cells ceases.

To obtain information concerning the intrinsic regulation of medullary blood-vessels, we first resorted to the method of direct microscopic observation which has been used by Forbes and his co-workers (1928) for similar studies in the parietal region. It proved to be comparatively easy to insert a suitable window in the normal occipito-atlantoid space, and to observe and measure changes in caliber of the pial vessels on the dorsal aspect of the medulla. This method was abandoned, however, because there was no way to decide whether the vessels in intimate relation with active nerve cells behaved like those of the pia. We have attempted to measure

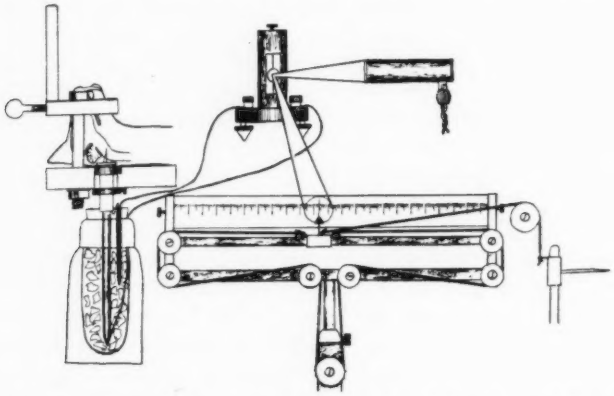

Fig. 1. Diagram of method used in measuring blood-flow in medulla of cat by cooled thermocouple.

arterial inflow by the method of Rein (1929), but have so far been unable to secure sufficiently repeatable *in vivo* calibrations to justify our using it for actual blood-flow measurements; the abundant anastomoses between the intracranial and extracranial circulations of the dog and cat would make the interpretation even of precisely measured arterial blood-flow changes extremely hazardous, as pointed out in an earlier publication (Schmidt, 1928a).

A suitable method was finally made available to us through the work of Gibbs (1933), who constructed a small thermocouple which could be inserted directly into the substance of the brain (or other tissue), circumventing the normally perfect thermal insulation of the central nervous system by setting up a heat gradient by means of an electrically heated wire close to the thermal junction. We have retained the principle of the

method but have simply reversed the direction of the heat gradient by mounting the thermal junction on a silver rod the other end of which was immersed in ice-water in a thermos flask. In this way the thermal junction was maintained at a temperature lower than that of the blood; changes in the amount of heat brought to it by the blood were reflected in an electrical current measured by a galvanometer and recorded graphically by a simple mechanical device.

The construction and arrangement of the apparatus which we have used are shown in figure 1.

The instrument to be described has proved rugged and adequately sensitive; possibly other dimensions or materials would improve its performance. It consists of a silver rod 23 cm. long and 6 mm. in diameter except at one end, where it is turned down to 3 mm. for a distance of 3 cm. This narrowed portion is covered with a hard-rubber sleeve with a wall 1 mm. thick, to serve as thermal insulation for the exposed part of the cooled rod; the free end of this sleeve is closed except for a small central hole through which the silver rod is continued as a no. 30 (B. & S. gauge) silver wire 6 mm. long. To the tip of this wire is soldered a no. 36 constantan wire; this is the active (hot) junction, and this is the part of the instrument that is thrust into the tissue. The constantan wire runs in a groove cut in the hard-rubber sleeve to the constant-temperature (cold) junction, which is embedded in paraffin-beeswax in a sealed glass tube. Both silver rod and glass tube are passed through a rubber stopper into a thermos flask of one pint capacity, which is filled with cracked ice and water; the hard-rubber insulated end of the silver rod protrudes. The silver wire of the cold junction and another silver wire soldered to the large end of the silver rod are connected to the galvanometer. We used a Leeds and Northrup moving coil galvanometer with a sensitivity of 4.5×10^{-8} ampere and a period of 4.4 seconds. With the circuit employed the galvanometer was slightly overdamped. The entire circuit had time constants such that the galvanometer reached a steady deflection within 5 seconds when the active junction was suddenly warmed or cooled. For convenience in operation a dry cell, connected through a rheostat and potentiometer, was provided to permit resetting of the image from the galvanometer at any desired point on the scale. A manually operated rider was kept in touch with the image on the recording scale, and a lever was thereby made to write upon the smoked paper of a kymograph. In all cases the writing lever moved up when the active junction was warmed (i.e., with increase in blood-flow); the graphic record followed the galvanometric deflection millimeter for millimeter. The scale was 34 inches from the galvanometer in all experiments. With ice-water (and cold junction) in the thermos flask, the galvanometric deflection was 145 mm. for each degree (C.) change in temperature, the curve being a straight line

over the investigated range (25–45°). The temperature of the active junction after insertion in the medulla was usually about 32°, as shown by the amount of dry-cell current required to bring the galvanometric image to a given point on the scale; rectal temperature of the animals was usually about 41°, so that the heat gradient was about 9°.

Substitution of an ice-bath for the electric heating circuit used by Gibbs (1933) made it possible to dispense with the entire heating circuit and with all insulation for the thermal junction. The part of the instrument inserted into the medulla could therefore be made smaller and more sensitive to changes in environmental temperature than would otherwise be the case; also, the danger of overheating the tissue and the possibility of escape of current from one circuit to the other were both eliminated. These advantages were secured at the expense of flexibility: with our instrument rigid fixation of the animal's head was essential. This was accomplished, as shown in figure 1, by clamping the animal's flexed head to two vertical bolts adjustable in a slot to heads of different sizes; the clamp was Palmer's standard head-holder, swung vertically from a heavy stand clamped to the animal table.

It was also necessary to provide a guide for proper direction and penetration of the instrument, as well as protection of the exposed medulla against drying. Both purposes were achieved by means of a hard-rubber tube 32 mm. long, with a bore which just allowed the hard-rubber insulated end of the instrument to pass easily; its walls were about 1 mm. thick; at one end it bore a flange 1 mm. deep and 9 mm. outside diameter. After exposure and excision of the occipito-atlantoid ligament and subjacent dura, with the animal lying on its abdomen and its head strongly flexed the flange of this tube was slipped under the occipital and atlas bones; a brass sleeve was pushed down the tube and fastened with a setscrew to prevent forcing the flanged end into the medulla. The animal was turned on its back (the opening of the tube being closed with wax to retain cerebrospinal fluid), the tube was passed through a suitable slot in the board under its head, and the head was clamped in position; another brass sleeve was passed over the tube into firm contact with the lower surface of the headboard and fastened with a setscrew as an additional precaution against forcing the tube into the medulla. The insertion of the instrument then followed simply by pushing its exposed part through the tube as far as it would go, after which the thermos flask was blocked firmly in place. The dimensions are such that the outer 3 to 4 mm. (i.e., the thermal junction and adjacent fine wires) were imbedded in the substance of the medulla but the thick parts of the rod could not touch it. The hard-rubber tube was forced into the central hole of the stopper of the thermos flask, thereby providing a water-tight joint and preserving the normal closed relations of the cerebrospinal circulation.

Other items in the procedure were as follows: Cats were used exclusively; narcosis was produced by sodium amytal (0.06 gram per kilo intraperitoneally); for decerebration, a ligature method (Schmidt, 1923) was used, with external carotids tied but internal and common carotids open; blood pressure was registered from a femoral artery using 25 per cent sodium thiosulfate as the anticoagulant; respiration was recorded, when desired, by a pneumograph or by the Lieb-Cushny (1912) plethysmograph; artificial respiration was provided by a double Meyer pump allowing a closed system with both pressure and suction.

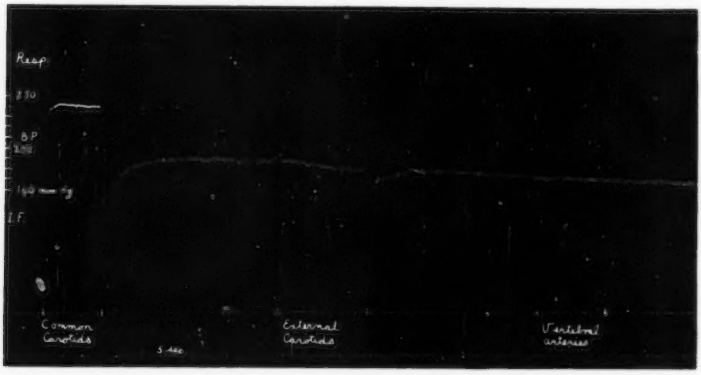

Fig. 2. Cat—amytal; natural respiration; vagi intact. Records, from above downward: pneumogram ("Resp."); femoral blood-pressure ("B-P"); indicated flow of blood through medulla ("I.F."); signal; time, which is zero level for blood-pressure. Between signals common carotid, external carotid and vertebral arteries were occluded in turn, release indicated by second signal. All tracings to be read from left to right. Calibrations at left refer to blood-pressure tracing; excursions of indicated flow in millimeters may be measured from same scale, dividing by two.

1. *Control experiments.* The deflections of the galvanometer produced by such influences as occlusion and release of the carotid or vertebral arteries or intravenous injection of adrenalin were uniformly such as to indicate sensitivity of the instrument to changes in blood-flow about the active junction. An illustrative set of examples is shown in figures 2 and 3. Carotid occlusion and adrenalin injection, as well as CO_2 inhalation (see figs. 5 and 8) were used routinely as tests for sensitivity. It seemed advisable, however, to supplement this presumptive evidence of competence of the instrument by a set of tests of its response to known changes in medullary blood-flow under controlled conditions. For this purpose four experiments were made in which the instrument was inserted in the usual manner in the medulla of a cat, the head being removed and perfused

with heparinized cat blood via the carotid arteries. Ether narcosis was used; preparation was made for insertion of the instrument through the occipito-atlantoid space; cannulas were inserted in both common carotids, directed headward; heparin was injected intravenously (0.1 gram per kilo) and the animal was bled to death; the head was quickly removed and fixed in the holder as usual, the instrument was inserted in the medulla,

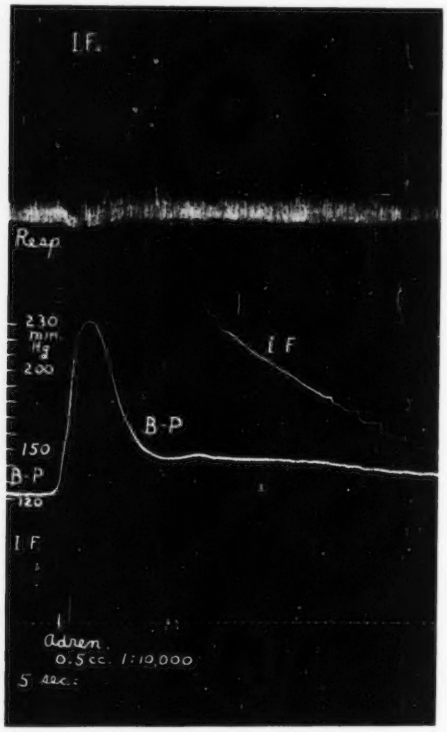

Fig. 3. Same experiment as figure 2; vagi cut. Arrangement and significance of records same as in figure 2. Adrenalin injected intravenously.

and perfusion was begun through the carotids by means of the Richards-Drinker pump; effluent blood was collected through the neck of a toy balloon, the cut outer end of which was slipped over the stump of the vertebral column and tied in place. In two experiments crossed-perfusion was used (i.e., the pump was fed with blood from a carotid of a heparinized donor cat, the effluent blood being returned to a jugular vein of the donor). In the other two freshly shed heparinized blood of cats was used; the blood

effluent from the head was aerated by means of a rotating aluminum disc driven by an electric motor inside a large glass jar. In the experiments with shed blood fluctuations in temperature were difficult to control.

In all four experiments the instrument at first responded properly to changes in blood-flow produced by changing the output of the perfusion pump. After some 30 minutes sensitivity decreased or disappeared, probably because of the development of cerebral edema, which was very evident at autopsy. The results obtained in the four experiments before sensitivity disappeared are shown in figure 4.

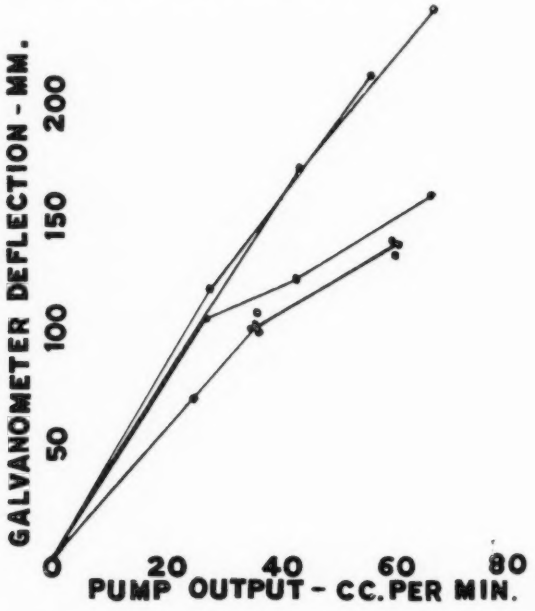

Fig. 4. Response of cooled thermocouple to changes in blood-flow in medulla of excised perfused heads of four cats.

We conclude from these experiments that the instrument is capable of detecting changes in blood-flow in the medulla.

It was also necessary to demonstrate, in connection with attempts to demonstrate vasomotor innervations in the medulla, that the procedures used in stimulating vasomotor nerves were adequate to produce changes that could be detected by the instrument. This was done in two experiments on cats, under amytal narcosis, in which the active junction was inserted successively in temporal and leg muscle, kidney, and spleen, with ice-water in the thermos bottle. Some of the results are illustrated in

figures 6 and 7; they furnished the necessary demonstration of adequacy of stimulation and competence of the instrument.

Although these tests convinced us that the method could be trusted to detect local vascular changes, it was necessary to exclude changes in temperature due to something other than changes in blood-flow before the results could be used for our purpose. Variations in temperature of the

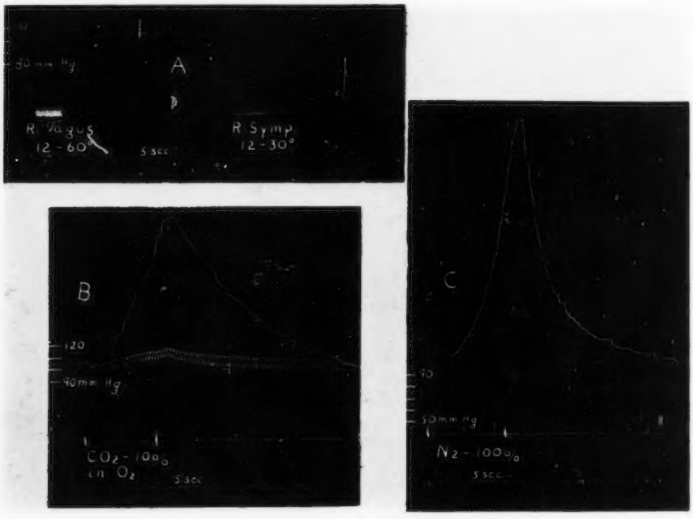

Fig. 5. Cat—amytal; curara; artificial respiration by double pump. Upper record—indicated blood-flow in medulla; lower record—femoral blood-pressure.

A—stimulation of central end of right vagus nerve with open platinum electrode, using Harvard inductorium (2 dry cells), secondary 12 cm. from primary and tilted to 60°. Stimulation of cephalic end of right cervical sympathetic in same way—secondary at 12 cm., tilted to 30°.

B—CO_2, 10 per cent in oxygen, run through air pump for 1 minute; 30 minutes after A.

C—pure nitrogen run through air pump for 1 minute.

Note that deflections of galvanometer were much greater in B and C than in A; record A shows greatest effects of nerve stimulation encountered in any curarized animal.

blood and changes in heat production in the vicinity of the active junction were considered in this connection. Body temperature was kept constant by means of heating lamps under the animal, rectal temperature serving as the guide; even when the animal's temperature was allowed to rise or fall a degree or more the galvanometric deflection was a slow, irreversible drift utterly unlike the rapid, reversible deflections produced by changes

in blood-flow. Changes in heat production by medullary cells could not be excluded in connection with each observation, but we were able to show that the procedures which elicited the most marked deflections when the thermal junction was kept cool (i.e., with ice-water in the thermos flask) produced no deflection when the heat gradient was abolished (i.e., with water at 40–42° in the flask). Thus, intravenous injection of adrenalin, inhalation of CO_2 or nitrogen, and carotid occlusion were without effect upon the galvanometer when the active junction was at blood temperature, but replacement of ice-water in the flask promptly restored the characteristic large deflections. If these deflections were due even in part to in-

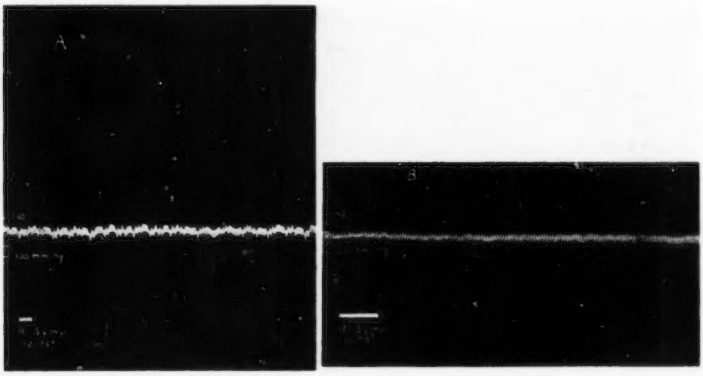

Fig. 6. Cat—amytal. Active junction in right temporal muscle, with ice-water and cold junction in thermos bottle.

A—natural respiration, before curara. Stimulation of cephalic end of right cervical sympathetic nerve as in fig. 5—secondary at 12 cm., tilted to 75°.

B—after curara (10 mgm. per kilo intravenously); artificial respiration; same stimulus as in A.

creased heat production by medullary cells the thermal junction should have disclosed that fact whether it was at 31° or 41°. We conclude that the results to be reported below are due mainly if not exclusively to changes in rate of blood-flow in the vicinity of the active junction.

We attempted to circumvent both general and local changes in metabolism by mounting the indifferent (cold) junction on the outer margin of the hard-rubber tip of the instrument, hoping that the hard-rubber would provide adequate thermal insulation from the cold silver core. Instruments so constructed were entirely insensitive. We also tried the cold junction in the animal's body (usually between the diaphragm and liver, occasionally in the pectoral musculature), so that changes in body temperature would have no effect upon the galvanometer. This procedure

was soon abandoned because changes in blood-flow about the cold junction simulated opposite changes in the medulla. Maintenance of the cold junction at a constant temperature in the ice-water of the flask was therefore preferred.

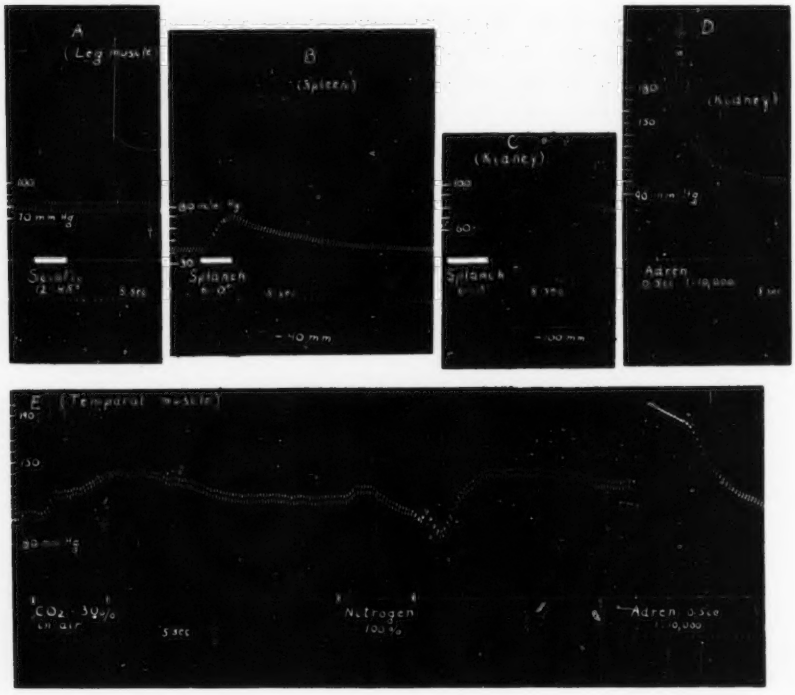

Fig. 7. Cats—amytal and curara; artificial respiration; records B and E from same experiment as figure 6, others from a second experiment. Ice-water and cold junction in thermos bottle.

A—active junction in muscles of right leg; right sciatic nerve cut—peripheral end stimulated as usual—secondary at 12 cm., tilted to 45°; slight twitch of leg at start of stimulation caused initial upward movement of indicated flow (upper record in each case).

B—junction in spleen; left splanchnic nerve stimulated with secondary at 6 cm., horizontal. Indicated flow fell 40 mm. below lower limit of recording system.

C—junction in left kidney; left splanchnic stimulated with secondary at 6 cm., horizontal. Indicated flow fell 100 mm. below lower limit of recording system.

D—same as C. Adrenalin injected intravenously.

E—junction in right temporal muscle; cervical sympathetics intact. CO_2, 30 per cent in air, and pure nitrogen, run through air pump for one minute. Adrenalin injection was intravenous.

Damage produced by insertion of the instrument into the medulla was negligible. After removal the point of insertion usually could not be detected even with a loupe. When the active junction was inserted into the medulla of a decerebrated cat the animal always gave a sudden start, but blood-pressure and respiratory movements were affected only momentarily, if at all. Under amytal narcosis the insertion had no visible effect whatever. Occasionally pial vessels were wounded in excising the dura; in such cases the instrument was always relatively insensitive.

When the instrument was used in the manner described above the galvanometer remained remarkably steady as long as conditions were constant. The rapidity and extent of the responses to given agencies (carotid occlusion, CO_2 or nitrogen inhalation, adrenalin injection, etc.) were quite variable from animal to animal. This is to be expected because the thermal junction was not inside a blood-vessel, and its sensitivity to changes in blood-flow must have depended upon the vascularity of the tissues surrounding it.

In the following sections we shall present the data obtained with this method as "indicated blood-flow," expressing them simply as deflection of the galvanometer in millimeters, starting from a convenient (and entirely arbitrary) control level. Calibration in terms of actual blood-flow was possible only by perfusion of the isolated head (fig. 4), the conditions of which are so far removed from those of the usual experiment that no attempt will be made to utilize the curves so obtained as quantitative calibrations.

2. *Vasomotor nerve influences.* Faradic stimulation of the cephalic ends of both cervical sympathetic and vagodepressor nerves was tried repeatedly in 20 cats under amytal narcosis. In 15 of them both carotid sinus nerves were stimulated and in 2 the nerve plexuses about both vertebral arteries were also stimulated. The vertebral stimulations were always without effect. In 3 of the animals cervical sympathetic stimulation caused a gradual and reversible fall in indicated flow without fall, or with actual rise, in blood-pressure; stimulation of the vagodepressor and sinus nerves also caused a rise in indicated flow without rise (or with an actual fall) in blood-pressure. The effects could be elicited repeatedly in these animals, and artificial respiration did not eliminate them. In no case however was the effect more than slight (the maximum was a 10 mm. fall with strong sympathetic stimulation); compared with the effect recorded with the instrument in muscle, spleen, or kidney when the vasomotor innervation was stimulated (figs. 6 and 7) even the greatest of these effects was insignificant.

In order to exclude a possible influence of narcosis upon a highly specialized (and therefore readily narcotizable) medullary vasomotor system, the same stimulations were repeated upon 5 decerebrate cats. The results

were never more striking than in the narcotized animals; in no case was there evidence of vasoconstriction or dilatation of sufficient intensity to imply physiological significance, and vertebral stimulation was entirely ineffective in the 2 decerebrated animals on which it was tested.

These results will not be considered any further for the following reason: in the last experiment of the series, in which the most marked (10 mm.) deflections had been obtained, we discovered accidentally that artificial respiration was not enough to prevent spurious shifts of indicated flow during nerve stimulation. We found that gentle pressure on the chest or electrical stimulation of the central end of a sciatic or anterior crural nerve would produce galvanometric deflections even more marked than those which followed stimulation of the vagodepressor and cervical sympathetic nerves, even though constant artificial respiration was being applied. Apparently changes in venous return from the head, resulting from changes in tone of respiratory muscles, were responsible. These observations led us to doubt the validity of the above-mentioned results, and we decided to repeat the stimulations in animals the tone of whose respiratory musculature could not change.

Two procedures were used for the purpose: curarization (10–15 mgm. per kgm. intravenously), 9 animals being used (5 decerebrates, 4 narcotized with amytal), and section of the spinal cord (between ligatures and without bleeding into the medulla) at the third cervical vertebra under ether anesthesia, which was then discontinued; five experiments of this sort were made.

The results were as follows: of the curarized animals, only one showed any trace of vasoconstriction following sympathetic stimulation or vasodilation with vagodepressor or sinus nerve stimulation. These results are shown in figure 5. Since there is evidence that curara in large enough amounts may weaken or paralyze vasomotor innervations (Boehm, 1920), we tested the response of the instrument, inserted in the temporal muscle of one of these animals, to cervical sympathetic stimulation before and after curara. The result (fig. 6) showed that although the vasomotor nerves in this tissue might have been somewhat weakened by the curara, they certainly were not paralyzed. In another animal the instrument showed intense vasoconstriction in the spleen and kidney upon stimulation of a splanchnic nerve after curara (fig. 7). In the one animal whose medullary vessels showed positive effects from nerve stimulation the response to CO_2 and anoxemia was much more marked than that to nerve stimulation (fig. 5).

The experiments in which the cord was cut enabled us to obviate spurious effects secondary to changes in tone of respiratory muscles without using curara. In 3 of the 5 there were slight (less than 10 mm.) decreases in indicated flow with sympathetic stimulation, and practically equal in-

creases with vagodepressor stimulation; the other animals gave negative results. In the positive experiments the results were difficult to interpret: stimulation of the sympathetic or vagodepressor of one side usually caused a decrease in indicated flow, while stimulation of the corresponding nerves on the other side caused an increase. In no case were the results of sympathetic stimulation opposite to those of vagus stimulation on the same

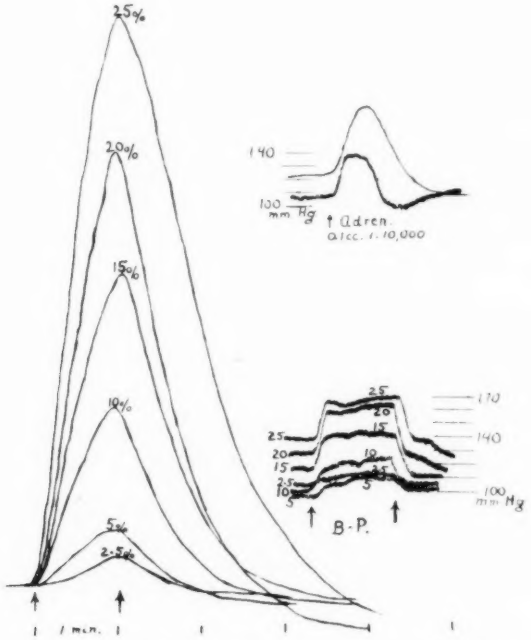

Fig. 8. Cat—amytal; natural respiration; active junction in medulla. CO_2 2.5, 5, 10, 15, 20 and 25 per cent in air (2 liters total volume in each case) rebreathed for one minute from rubber bag attached directly to tracheal cannula. Records of indicated flow superimposed at left, those of blood-pressure at right.

Maximum rise in blood-pressure (with 20 per cent CO_2) was 30 mm. Effect of an equal rise (30 mm.) produced by intravenous injection of adrenalin is shown at upper right. Rise in indicated flow was obviously not entirely due to rise in blood-pressure.

side. Carotid sinus stimulation (applied directly to the exposed sinus, the nerve being untouched) had only passive effects.

In addition to these nerve stimulations, intravenous injections of adrenalin were made in practically every experiment. In no case was there any evidence of constriction of medullary vessels; the curve of indicated flow followed that of blood-pressure quite faithfully. In two experi-

ments adrenalin was injected into a vertebral artery via the subclavian, and in one cephalad in each common carotid successively. Here again there was no trace of primary vasoconstriction in the medulla, the curve of indicated flow simply following that of blood-pressure.

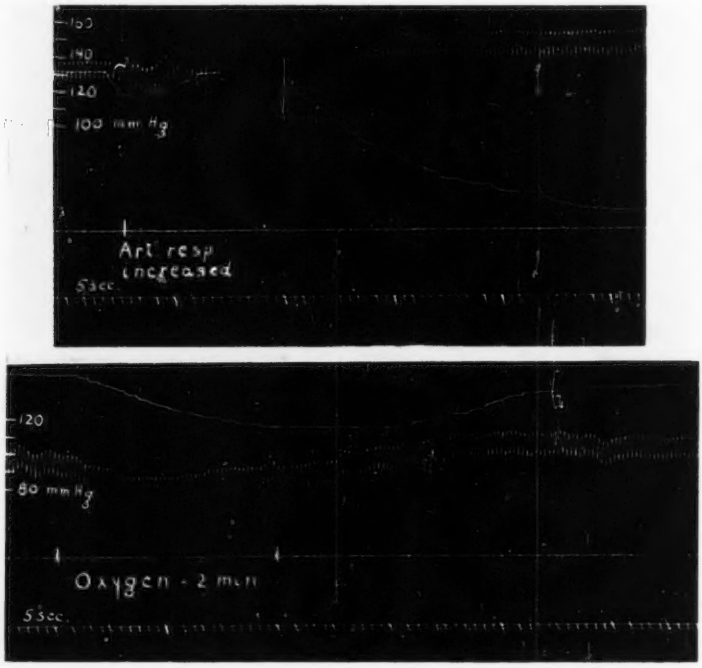

Fig. 9. Cat—amytal and curara; active junction in medulla; artificial respiration by double pump.

Upper record—thrust of air pump increased at signal. Note rise in blood-pressure with fall in indicated flow.

Lower record (another experiment)—pure oxygen run through pump for two minutes. Blood-pressure fell from 98 to 80, then rose to 88 during insufflation of oxygen; it had risen to 100 before indicated flow began to rise and flow remained below initial level when blood-pressure was 104–106.

We conclude that the vasomotor innervation of the blood-vessels of the medulla of the cat is so weak and inconstant that it is physiologically unimportant. We believe that a potent vasomotor influence would have been disclosed, if not by the nerve stimulations, at least by the action of adrenalin injected intra-arterially, or by the vasomotor excitation produced by asphyxia. We found that when the vasomotor center was

stimulated by CO_2-excess or oxygen-lack the central stimulant effect overshadowed the peripheral dilator action in a tissue with competent vasomotor innervation (fig. 7 E); in the medulla, however, both CO_2-excess and oxygen-lack produced only vasodilatation (see the following section).

3. *Chemical influences.* Although a potent nervous regulation of medullary blood-vessels could not be demonstrated, a remarkably effective chem-

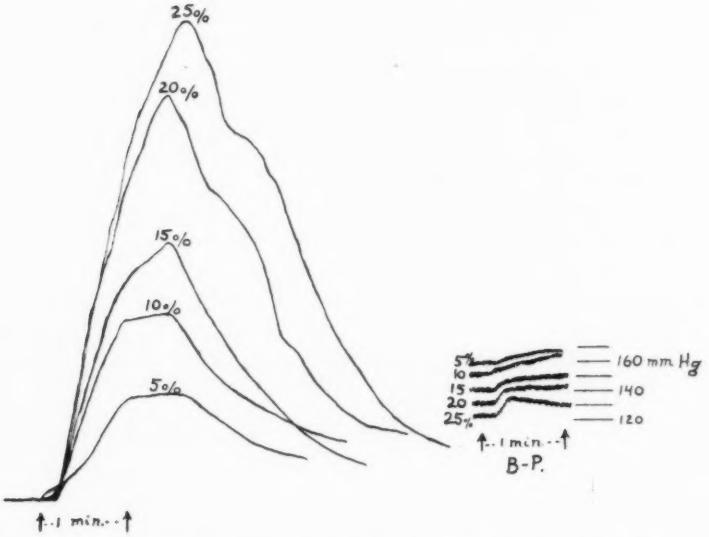

Fig. 10. Cat—amytal and curara; active junction in medulla; artificial respiration by double pump (closed system); CO_2 5, 10, 15, 20, and 25 per cent (2 liters of each) run through air pump for one minute. Records of indicated flow superimposed at left, those of blood-pressure at right.

Blood analyses: Control—CO_2 37.06 per cent, O_2 12.19 per cent; 5 per cent—CO_2 38.66 per cent; O_2 12.43 per cent; 10 per cent—CO_2 40.94 per cent, O_2 12.42 per cent; 15 per cent—CO_2 42.50 per cent, O_2 11.69 per cent; 20 per cent—CO_2 44.41 per cent, O_2 11.04 per cent; 25 per cent—CO_2 45.68 per cent, O_2 10.47 per cent.

ical regulation was constantly evident. Inhalation of CO_2 produced changes in indicated blood-flow comparable with those resulting from intravenous injection of adrenalin. Inhalation of nitrogen was also effective, but usually less so than CO_2. In the opposite sense, excessive artificial ventilation always, and oxygen inhalation occasionally, caused a decrease in indicated blood-flow. Examples of these effects are shown in figures 5, 8, and 9. Stimulation of sympathetic, vagus, or sinus nerves never elicited responses nearly as marked as these, and reactivity to

chemical influences was present in all the preparations that were completely negative to nerve stimulation.

The constancy and apparent effectiveness of this chemical regulation led us to investigate it further. We found that the degree of the response was proportional to the concentration of CO_2 in the inspired air or blood (fig. 8). We also found that the responses of curarized animals were of the same sort as those of spontaneously breathing ones (fig. 10); changes in respiratory activity were therefore not responsible for the observed phenomena. Increase in heat production by the medulla was excluded

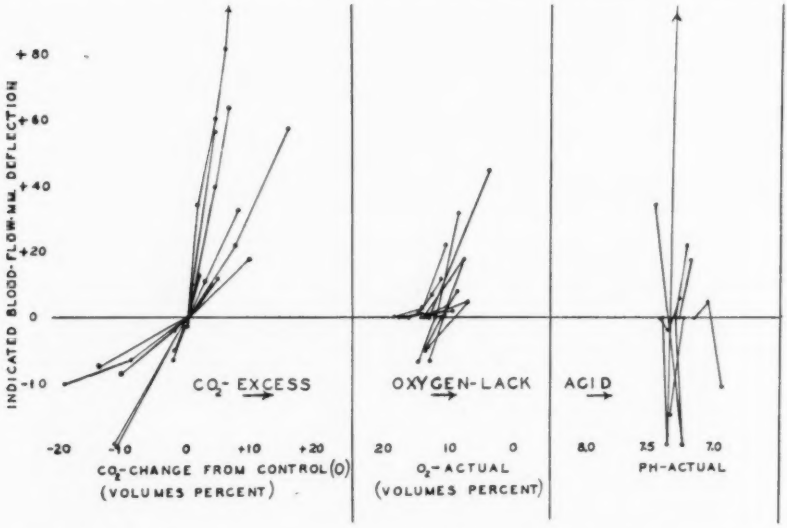

Fig. 11. Effects of alterations in arterial CO_2, O_2, and pH upon indicated blood-flow in medulla.

Data from 29 experiments upon curarized cats under amytal narcosis. Explanation in text.

by absence of galvanometric deflection during CO_2 insufflation when the thermos bottle was filled with water at 40°C.; substitution of ice-water led to the normal increase in indicated blood-flow during administration of CO_2. Rectal temperature was found to be unchanged during inhalation or insufflation of CO_2.

These observations convinced us that increase in CO_2 content of the blood has a very marked dilator influence upon medullary vessels. We next investigated the specificity of CO_2 in this connection, comparing its effectiveness with that of other acids and with oxygen-lack. For this purpose curarized cats under amytal narcosis were used. Blood samples

were drawn from a femoral artery; pH estimations were made by the colorimetric method of Hawkins (1923) and oxygen and CO_2 content were determined by the manometric method of Van Slyke and Neill (1924). Gas mixtures were administered through a double air pump (closed system); acids (HCl, lactic, ammonium chloride) and alkali (sodium bicarbonate) were injected intraperitoneally or intravenously. Twenty-nine experiments were made.

We have attempted to portray the findings graphically in fig. 11, so as to show the comparative uniformity, degree, and threshold of sensitivity of the responses of indicated medullary blood-flow to progressive increases and decreases of the CO_2, oxygen, and hydrion concentration of the blood. The points represent the maximum blood-flow effect of a given change in the blood, and the lines connect observations made in a single experiment; in all cases the control reading for blood-flow is placed on the zero line. The CO_2 results are plotted in terms of change in blood CO_2 from the control level and not of actual CO_2 content because, these being curarized animals under artificial respiration which was adjusted so as to secure the best possible circulatory conditions, blood CO_2 content was very variable (i.e., from about 15 vols. per cent to 45 vols. per cent) during the control period in different animals. In the cases of oxygen and pH the deviations were naturally much smaller and the data are plotted as found. The general arrangement is the same in all three: the curves should slope toward the right if medullary blood-flow is increased by CO_2-excess, anoxemia, or acidosis; the steepness of the slope indicates the sensitivity. The parts of the curves above the zero line represent the effects of insufflation of CO_2 (from 1 to 30 per cent in air) or nitrogen (from 25 to 100 per cent in air) for one minute; the pH curves show the effects of fixed acid or alkali only, pH changes produced by CO_2 or nitrogen being excluded. The parts of the curves below the zero line show the effects of increased artificial respiration (CO_2 curves), or insufflation of oxygen (oxygen curves), or decrease in blood-flow following absorption of fixed acid or alkali (pH curves). Changes in blood-pressure are not taken into account in these graphs; such changes were slight (less than 10 mm. Hg) in the cases of CO_2 and oxygen shifts unless they were extreme (i.e., insufflation of CO_2 of 20 to 30 per cent, or nitrogen above 75 per cent); when blood-pressure was markedly reduced by acid or alkali the results were discarded. While changes in blood-pressure undoubtedly played a part in many of the changes in blood-flow following these changes in the blood, it was frequently possible to secure the blood-flow change without corresponding change in pressure. To illustrate these points data from representative experiments are shown in table 1.

These results lead us to believe that CO_2 is a specific regulator of the tone of medullary blood-vessels. Its effects were more uniform, more

TABLE 1

DATE	CONDITIONS	TIME	BLOOD PRESSURE		INDICATED FLOW	BLOOD		
			From	To		CO_2	O_2	pH
			mm. Hg	*mm.* Hg	*mm.*	*vols. per cent*	*vols. per cent*	
5–18–33	Control	12:10	142		0	34.08	20.57	7.35
	CO_2 10% in air—1 min.	12:15	130	142	+18	43.50	20.70	7.30
	CO_2 20% in air—1 min.	12:25	126	148	+57	48.90	19.43	7.2
	Recovery—control	12:34	130		0	31.85	20.02	7.15
	NH_4Cl 2 cc. 20% i.p.	12:49	140	154	+5			
	Acid effect	12:54	148		−13	23.42	19.19	7.05
	NH_4Cl 3 cc. 20% i.p.	12:59	144	144	−13			
	Acid effect	1:04	130		−21	13.78	19.41	6.95
5–31–33	Control	10:39	146		0	35.37	11.21	7.41
	NH_4Cl 9 cc. 20% i.p.	10:43	140	148	+10			
	Acid effect	10:53	142		−39	26.98	11.71	7.36
	Acid effect	10:59	142		+224	23.16	12.75	7.31
6–1–33	Control	11:44	152		0	38.44	19.31	7.31
	NH_4Cl 10 cc. 25% i.p.	11:48	156	174	+5			
	Acid effect	11:54	146		−12	28.06	19.60	7.21
6–7–33	Control	12:33	104		0			7.47
	CO_2 1% in air—1 min.	12:35	100	102	+9			7.47
	Control	12:51	110		0			7.40
	CO_2 1% in air—1 min.	12:54	110	110	+12			7.40
	Control	1:06	128		0			7.37
	CO_2 2% in air—1 min.	1:10	120	120	+5			7.35
	Control	1:26	118		0			7.32
	CO_2 5% in air—1 min.	1:28	116	116	+8			7.30
	Control	1:46	98		0			7.32
	CO_2 10% in air—1 min.	1:47	98	108	+35			7.28
	Control	2:16	88		0			7.27
	CO_2 3.3% in air—1 min.	2:18	84	84	+18			7.27
6–14–33	Control	11:01	152		0	29.31	17.43	7.37
	Nitrogen 25% in air—1 min.	11:05	142	144	0	29.26	16.62	7.30
	Nitrogen 30% in air—1 min.	11:21	134	138	+1	26.10	15.02	7.34
	Nitrogen 50% in air—1 min.	11:34	110	112	+1	23.13	15.45	7.26
	Nitrogen 75% in air—1 min.	11:56	90	82	+12	21.94	11.87	7.26
6–20–33	Control	2:27	136		0	40.06	16.55	7.37
	CO_2 5% in air—1 min.	2:30	140	144	+13	41.42	16.37	7.37
	Increased ventilation. Control	2:49	168		0	35.35	18.48	7.37
	Nitrogen 50% in air—1 min.	3:02	182	184	+3	34.50	15.01	7.35
	Nitrogen 75% in air—1 min.	3:10	158	112	+22	33.85	10.98	7.39
	Recovery—Control	3:27	148		0	31.05	18.35	7.37

TABLE 1—*Concluded*

DATE	CONDITIONS	TIME	BLOOD PRESSURE		INDICATED FLOW	BLOOD		
			From	To		CO_2	O_2	pH
			mm. Hg	*mm.* Hg	*mm.*	*vols. per cent*	*vols. per cent*	
6-20-33	CO_2 5% in air—1 min.	3:34	134	134	+12	35.48	17.88	7.33
	CO_2 10% in air—1 min.	3:39	124	126	+33	38.59	16.76	7.29
	Recovery—control	3:55	142		0	29.75	17.36	7.38
	HCl 2 cc. 0.1N. i.v.	4:02	128	140	+6			
	Acid effect	4:03	130		−4	27.80	17.14	7.35
	HCl 4.4 cc. 0.1N. i.v.	4:14	110	122	+10			
	Acid effect	4:16	110		+10	24.54	16.54	7.33
	HCl 10 cc. 0.1N. i.v.	4:25	94	108	+29			
	Acid effect	4:27	66		+6			7.27

powerful, more nearly proportional to the change in the blood, and the threshold of sensitivity was vastly lower than was the case with the other agents tested. Thus, indicated blood-flow was sometimes altered by changes in blood CO_2 amounting to less than one volume per cent, the actual CO_2 content appeared to make no difference, a change in either direction having the usual effect over the entire range studied (from about 15 to 45 vols. per cent). This effect of CO_2 was not due simply to its acidity because we were frequently able to produce increases in indicated flow by administration of weak (1 per cent) CO_2-air mixtures without readable change in pH (table 1). Furthermore, quite marked shifts in pH following fixed acid or alkali gave additional reason to ascribe a specific rôle to CO_2, for indicated blood-flow showed a tendency to follow the change in CO_2 rather than the change in pH (fig. 11 and table 1). With regard to oxygen-lack, the effects upon indicated flow were uniform in direction, but nearly always they were less marked and the threshold of sensitivity was decidedly higher than was the case with CO_2. The tendency of the oxygen curves to become horizontal over the range of blood oxygen from 18 to 12 vols. per cent (fig. 11) illustrates a regular finding: the medullary vasodilator effect of anoxemia began to become evident only when blood oxygen had fallen to about 12 vols. per cent. From this point on the dilator effect progressed with decreasing oxygen, but even with blood oxygen reduced to less than 10 vols. per cent the increase in indicated flow was usually less marked than that produced by an increase of about 5 vols. per cent in blood CO_2. In no case was the maximal increase in indicated flow produced by anoxemia (down to 5 vols. per cent arterial oxygen) as marked as the maximal effects of CO_2 excess; it is probable that the cardiac depressant effect of extreme anoxemia is concerned in this particular comparison but the relative thresholds of sensitivity remain undisturbed.

The threshold to changes in pH produced by fixed acid or alkali proved impossible to establish with any certainty because of the capriciousness of the effects upon indicated flow. In only one instance (table 1, second experiment) was there a marked increase in indicated flow associated with fall in pH; here, following intraperitoneal injection of ammonium chloride, indicated flow fell as pH fell from 7.41 to 7.36, but shortly afterward indicated flow rose sharply; a blood sample then showed a pH of 7.31. According to this, the threshold to pH changes would lie in the neighborhood of a 0.1 shift.

Although the scope of these experiments was to have been limited to the normal intrinsic regulation of the medullary circulation and drug actions were of subsidiary importance, we made a number of observations of the latter sort which are worth mentioning at this time. We found that a number of agents which are either constrictor or without conspicuous effects upon blood-vessels elsewhere in the body were capable of producing marked increases in indicated blood-flow in the medulla. This group included adrenalin, ephedrine, pituitrin, morphine, ether, and hypertonic sodium chloride and glucose; ether was particularly effective in producing enormous medullary vasodilatation without change in blood-pressure. Vasodilators such as nitroglycerine, amyl nitrite, acetyl choline, and caffeine also produced some medullary vasodilatation, but they seemed to be decidedly less effective than CO_2.

Discussion. The chief conclusion to be derived from these experiments is this: the blood-vessels of the medulla of the cat, narcotized with amytal or decerebrated, have a highly developed intrinsic chemical regulation; they may be subject to some influence from vasomotor nerves, but this is so slight as to be neglibible when contrasted with the potency of the chemical regulation of medullary vessels and the intensity of vasomotor influences in other parts of the body. This confirms the results of our earlier experiments (1928). It is also in accord with the attitude recently taken by Forbes, Finley, and Nason (1933) as a result of extensive experience with the responses of the pial vessels of the parietal area of the cat. It is further supported by the fact that stimulation of the vasomotor centers by insufflation of CO_2 or nitrogen caused only dilatation of medullary vessels, whereas vasoconstriction in tissues under competent vasomotor control could be detected by our instrument under similar conditions (fig. 7 E). Because our measuring device was in intimate contact with the nerve cells of the medulla, and because we took precautions to exclude adventitious influences such as changes in tone of respiratory muscles and changes in muscular activity, we feel satisfied that the results give a fair picture of the actual regulation of the blood-vessels of the medulla.

A second conclusion is this: the normal intrinsic regulation of medullary vessels is accomplished mainly if not exclusively by a specific dilator

influence of CO_2. The effects of alterations in blood CO_2 were so uniform, so striking, so nearly proportional to the change in CO_2, and the threshold of sensitivity was so low even under the unavoidably abnormal conditions of the experiments, that we regard them as manifestations of a normal regulation of outstanding physiological importance. The implications of this statement will not be discussed at this time; redistribution of blood among different parts of the central nervous system in accordance with their functional activity could be accomplished in this way, but more information is required before this can be regarded as more than a plausible guess. We hope to secure the necessary information. The influence of oxygen-lack appears to represent a toxic action having decidedly less physiological importance than that of CO_2; the same is true of changes in pH. If there are other hormonal influences we did not discover them; acetyl choline is excluded because atropine had no consistently constrictor effect upon medullary vessels; we have not tested histamine.

A third point of considerable interest is the abundance of measures which appear to produce marked dilatation of medullary vessels, compared with the paucity of constrictor agencies. So far we have been able to produce marked and repeatable medullary vasoconstriction only by excessive artificial respiration (meaning reduction in blood CO_2), or by insufflation of oxygen after anoxemia; of these the former was the more powerful and uniform. This suggests that the medullary vessels normally have a high intrinsic tone which is antagonized to varying degrees by the dilator effect of CO_2 in particular. Compared with vascular areas under competent nervous regulation, in which vascular tone is entirely dependent upon vasomotor nerve influences possibly supplemented by vasoconstrictor hormones, the situation in the medulla would appear to be exactly the reverse: an intrinsic high tone is variably antagonized by chemical vasodilator influences of which CO_2 is outstanding.

As a result of these experiments we regard the existence of an intrinsic chemical regulation of medullary blood-vessels of the cat as established beyond reasonable doubt. The nature of this regulation is such as to assist in the maintenance of a constant chemical environment within the cells of the medulla. The physiological significance of these relationships, suggested by us in 1928, subsequently denied by Heymans and Bouckaert (1930), reaffirmed in somewhat modified form by us in 1932, and again denied by Bouckaert and Heymans (1933), remains to be decided. Certainly the existence of a normal intrinsic chemical regulation must be taken into account in experiments of the sort just alluded to. We have already suggested (1932b) that in Heymans' experiments medullary blood-vessels may have been more constricted than in ours, but have not yet had the opportunity to investigate the point.

Finally, a direct and marked influence by the carotid sinus or vago-

depressor nerves upon the tone of blood-vessels in the medulla was not demonstrable in our experiments. The various effects of sinus excitation certainly cannot be explained as the result of a primary increase in blood-flow through vegetative centers in consequence of a primary marked dilatation of the blood-vessels supplying the medulla—a conception prominent in our minds when we undertook these experiments. We found no evidence of the intense cerebral vasoconstriction as a result of sinus excitation which Weiss and Baker (1933) have recently postulated in certain human subjects.

These studies are being extended to other parts of the brain, beginning with the hypothalamic region. Evaluation of the results and discussion of the literature will be postponed until the studies have been completed.

SUMMARY AND CONCLUSIONS

1. By means of a small thermocouple which was inserted directly into the tissue and which was maintained at a lower temperature than the tissue through cooling of the stem on which it was mounted, changes in blood-flow were studied in the medulla of anesthetized and decerebrate cats.

2. A competent vasomotor nerve control over medullary vessels was not revealed by stimulation of vagodepressor, cervical sympathetic, carotid sinus, or perivertebral nerves, by intravenous or intravertebral injection of adrenalin, or by asphyxia.

3. A definite and powerful regulation of these vessels by CO_2 excess and oxygen-lack was constantly evident. The influence of CO_2 appeared to be practically specific; pH changes were uncertain in their effects, and anoxemia was effective only when it was intense.

4. Many agents apparently dilated medullary vessels but only one—excessive ventilation—seemed capable of contracting them definitely and regularly. It is suggested that medullary vessels have an intrinsically high tone that is continuously antagonized by vasodilator metabolites, CO_2 being outstanding. The intrinsic regulation of medullary vessels is different from that of other vessels in that it depends upon variable dilatation rather than constriction, and upon a normal metabolic product rather than upon vasomotor nerve influences or vasoconstrictor hormones.

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